CHARACTERIZATION OF ENDOCRINE DISRUPTING POTENTIALS OF MUNICIPAL EFFLUENTS FROM SIX WASTEWATER TREATMENT PLANTS ACROSS CANADA

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ABSTRACT

Over the past three decades, concerns have been raised regarding the potential adverse effects of certain natural and synthetic chemicals that can disrupt the endocrine systems of humans and wildlife. These endocrine disrupting chemicals (EDCs) have been reported to cause developmental and reproductive effects at low concentrations (ng/L) in many vertebrate species, particularly in aquatic organisms such as fish. One of the most prevalent sources of EDCs in aquatic environments is municipal wastewater effluents (MWWEs). This is because conventional wastewater treatment systems are inefficient at removing many of the diverse contaminants present in raw sewage, including EDCs. Although multiple initiatives have been initiated to establish standardized testing and monitoring criteria for EDCs in the environment worldwide, our understanding of the contribution of MWWEs to endocrine disruption in Canadian surface waters is incomplete. Therefore, the main aims of this project were to 1) further our understanding of the contribution of MWWEs to the contamination of freshwater bodies in Canada with EDCs, and to 2) characterize the removal efficiency of EDCs by six wastewater treatment plants (WWTPs) across Canada. Specifically, this study explored the presence of EDCs and their potencies in influents and effluents as a function of wastewater treatment level/system, climate/seasonality and population size served by the WWTP using a combination of three *in vitro* bioassays and targeted chemical analysis. The MVLN, MDAkb2, and H295R Steroidogenesis assays were applied to assess (anti-)estrogenic, (anti-)androgenic and steroidogenesis disrupting potentials, respectively, of extracts of influents and effluents collected throughout the year from the WWTPs of the cities of Saskatoon (SK), Regina (SK), Guelph (ON), Kitchener (ON), Quebec City (QB) and Montreal (QB). In parallel, targeted chemical analysis was performed to determine the presence of selected chemicals with proven or suspected endocrine activities, and results were correlated with bioactivities determined

in vitro. Overall, influents showed great androgenic activities regardless of treatment plant while significant estrogenic potentials were only observed in a few cases such as Regina effluent and Montreal influent. With the exception of Montreal, high to moderate treatment efficiencies of WWTPs occurred for the removal of androgens, while low or no removal of substances with estrogenic properties was observed. Significant anti-estrogenic and anti-androgenic potentials were detected in most of the influent and effluent samples, regardless of the treatment level. In general, WWTPs representing less advanced treatment technologies were less efficient at removing certain endocrine active substances. In particular, effluents from the two lagoon-based facilities, Regina and Montreal, had significant remaining estrogenic and androgenic activities, respectively. Furthermore, population size seemed to play an important role regarding EDC removal, with WWTPs serving greater than 500,000 habitants showing decreased removal of compounds with endocrine activities in general. However, given the limited sample size (only two of the cities investigated had populations greater than 500,000 inhabitants) it cannot be determined with certainty whether this decreased removal efficiency was a result of population size or simply insufficient capacities of the WWTPs. Thus, additional studies including more treatment facilities with different treatment levels and larger population sizes should be conducted to determine whether population does significantly affect the removal of EDCs. Furthermore, our original hypothesis that extremely cold temperatures would result in decreased efficiency of EDC removal due to reduced biological activity and light exposure was not always supported by the results. Samples collected during the spring season had the highest endocrine activities overall, which could potentially be a result of colder months. However, neither early nor late winter samples showed a comparable effect on removal efficiency. The observation that spring samples had the greatest endocrine activities may be of significant ecological concern as this season coincides with

the spawning season of many fishes. This concern was further corroborated by two parallel studies that investigated the impacts of MWWEs collected from the Regina and Saskatoon WWTPs on fish. These studies observed general inhibition of reproductive functions such as delayed maturation, degeneration of gonadal tissues, reduction in the expression of secondary sex characteristics, and significant reduction of fecundity in fathead minnows exposed to both diluted effluents or that were collected downstream of the WWTP outflow of Regina. The observation that antagonistic effects at both the ER and AR represented the most prevalent endocrine potentials was also supported by chemical analysis that revealed greater concentrations of compounds with the ability to act as ER and AR antagonists, while there were low concentrations or no presence of chemicals previously shown to agonistically interact with these receptors. The results obtained by the combination of *in vitro* and the two parallel *in vivo* and chemical analysis demonstrated that *in vitro* assays can be used as a cost-effective tool for prioritizing potential endocrine disrupting impacts of MWWEs in aquatic environments. The significant endocrine activity, in particular, antagonism of sex steroid receptors, warrants further investigations to characterize the actual risks they may pose to aquatic wildlife. This is particularly true in cases where WWTPs utilize primary and/or outdated lagoon-based treatment technologies, such as Regina and Montreal. Furthermore, in cases where effluent flow is proportionally higher than that of the receiving water body, which can be encountered in many urban municipalities in semi-arid regions such as Regina in southern Saskatchewan, or in situations where the population is greater than WWTPs' treatment capacity, bypassing untreated sewage, downstream ecosystems may be of particular risk.

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TABLE OF CONTENTS

LIST OF FIGURES

expressed as fold-change relative to the positive control (PC; 36.3 ng/L DHT). Data are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$).

Figure 2.4 - Changes in 17β-estradiol production caused by extracts of influents and effluents (1x concentrated) from Regina (A) and Saskatoon (B) WWTPs sampled during four seasons in 2014/2015 determined by H295R Steroidogenesis Assay. Data are relative changes compared to the solvent control (SC). Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC ($p<0.05$). FOR forskolin [10 μM]. PRO prochloraz [3 μM]. *$p<0.05$.

Figure A2.1 - Example of dose-response expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents at 0.1x, 0.3x, 1.0, 3.0x and 10x concentrations from Regina during spring determined by MDA-kb2 in vitro Assay. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A2.2 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by MVLN in vitro Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A2.3 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by MDA-kb2 in vitro Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A2.4 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by H295R Steroidogenesis Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure 3.1 – Geographical location (in green), treatment level of the six WWTPs studied across Canada. Data on population (pop) served are according to Statistics Canada, Census Profile, 2016.

Figure 3.2 - Estrogenic (A and B) and anti-estrogenic (C and D) activity of extracts of influents and effluents (1x concentrated) collected from 6 WWTPs during spring (A and C) and summer (B and D) of 2014 determined using the MVLN *in vitro* assay. Estrogenicity is expressed as relative changes compared to the solvent control (SC). Anti-estrogenic activity is expressed as fold-change relative to the positive control (PC; 81.7 ng/L E2). Data are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$). Data for Regina and Saskatoon are from chapter 2.

Figure 3.3 - Androgenic (A and B) and anti-androgenic (C and D) activity of extracts of influents and effluents (1x concentrated) collected from 6 WWTPs during spring (A and C) and summer (B and D) of 2014 determined using the MDA-kb2 *in vitro* assay. Androgenicity is expressed as relative changes compared to the solvent control (SC). Anti-androgenic activity is expressed as fold-change relative to the positive control (PC; 36.3 ng/L DHT). Data are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$). Data for Regina and Saskatoon are from chapter 2.

Figure 3.4 – Changes in 17β-estradiol production caused by extracts of influents and effluents (1x concentrated) from 6 WWTPs sampled during spring (A) and summer (B) of 2014 determined by

H295R Steroidogenesis Assay. Data are relative changes compared to the solvent control (SC). Results are represented as the mean ± SEM (n=4). The asterisk (*) denotes a significant difference from the SC ($p<0.05$). FOR forskolin [10 μM]. PRO prochloraz [3 μM]. *$p<0.05$. Data for Regina and Saskatoon are from chapter 2.

Figure A3.1 - Example of dose-response expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents at 0.1x, 0.3x, 1.0, 3.0x and 10x concentrations from Regina during spring determined by MDA-kb2 in vitro Assay. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.2 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MVLN in vitro Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.3 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MVLN in vitro Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.4 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MDA-kb2 in vitro Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.5 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by

MDA-kb2 in vitro Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.6 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by H295R in vitro Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Figure A3.7 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by H295R in vitro Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

LIST OF TABLES

Table A3.1: Characteristics of all six WWTPs in Canada. Information obtained through a collaboration between WWTPs' personnel and the AIME project. Concentrations below than Method of detection limit (<MDL). Data not available (-).

Table A3.2 – Summary of analytical results detected by Orbitrap LC-MS expressed as ng/L in samples of influents and effluents from six WWTPs across Canada during spring and summer of 2014 for 19 emergent contaminants. Concentrations below the method detection limit (<MDL). Samples not available for chemicals analysis (N/A).

Table A3.3: Extraction recovery (%) of spiked water samples from influent and effluent. Each sample was spiked in triplicate, average results are shown (n=3).

Table A3.4 - Chemical standard used for Orbitrap analysis and sample spikes

LIST OF ABBREVIATIONS

°C – Degrees Celsius

µg – Microgram

µg/L – Microgram per Liter

µM - Micromol

11-keto – 11-Ketotestosterone

ACN - Acetonitrile

AEQ - Androgen Equivalency

ANOVA - Analysis of Variance

AR - Androgen Receptor

ARE - Androgen Response Element

ATCC - American Type Culture Collection

AhR - Aryl hydrocarbon Receptor

BPA - Bisphenol-A

CMA - Census Metropolitan Area

CV - Coefficient of Variation

DCM – Dichloromethane

DCC-FBS - Dextran-charcoal FBS

DDE - 1,1-Dichloro-2,2-bis(p-chlorophenyl)-ethylene

DDT - Dichloro-diphenyl-trichloroethane

DEET - N,N-Diethyl-meta-toluamide

DHT – Dihydrotestosterone

DMEM-F12 - Dulbecco's Modified Eagle Medium-F12

DMSO - Dimethyl Sulfoxide

E1 - Estrone

E2 - 17β-estradiol

EE2 – Ethynylestradiol

E3 - Estriol

EC - Effective Concentration

ECCC - Environment and Climate Change Canada

EDA - Effect Directed Analysis

EDC - Endocrine Disrupting Chemical

EDSP - Endocrine Disruptor Screening Program

EEQ - Estrogen Equivalency

ELISA - Enzyme-linked Immunosorbent Assay

ER - Estrogen Receptor

ERE - Estrogen Response Element

EDSP - Endocrine Disruptor Screening Program

FBS - Fetal Bovine Serum

FHM - Fathead Minnow

FOR – Forskolin

g - Gram

GR - Glucocorticoid Receptor

h - Hour

HF - Hydroxyflutamide

HLB - Hydrophilic-lipophilic Balance

HT – Hydroxytamoxifen

L - Liter

LC-MS - Liquid Chromatography-Mass Spectrometry

LYES - Yeast Estrogen Screen, including a digestion step with the enzyme Lyticase

mL – Milliliters

m^3 – Cubic Meter

min - Minutes

MCX - Mixed Cation-exchange

MDL - Method Detection Limit

MWWE - Municipal Wastewater Effluent

n = Sample Size

ng – Nanogram

nM - Nanomolar

NP – Nonylphenol

NSAIDs - Nonsteroidal Anti-inflammatory Drugs

OP - Octylphenol

OECD - Organization for Economic Co-operation and Development

P – Progesterone

pM – Picomolar

PBDEs - Polybrominated Diphenyl Ethers

PBS - Phosphate Buffered Saline

PC - Positive Control

PCBs - Polychlorinated Biphenyls

Pop – Population

PPCPs - Personal Care Products

PRO – Prochloraz

SEM - Standard Error of the Mean

SC – Solvent Control

SIM - Selected Ion Monitoring

SPE - Solid Phase Extraction

T - Testosterone

US-EPA – United States Environmental Protection Agency

UHPLC - Ultra-high-performance liquid chromatography

UV – Ultraviolet

$\text{v/v} = \frac{volume\ soluble}{volume\ of\ solution} x\ 100$

VTG - Vitellogenin

WWTP - Wastewater Treatment Plant

YAS - Yeast Androgen Screen

YES - Yeast Estrogen Screen

PREFACE

This thesis is written in manuscript style format. Chapter 1 is a general introduction and literature review of endocrine disrupting chemicals, their occurrence, and sources, known effects on aquatic life, as well as methods of detection, and also provides a brief review of the concept of effect-directed analysis. Chapters 2 and 3 are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 4 is a general discussion and synthesizes the findings of Chapters 2 and 3 and relates them to the overall objectives this thesis set out to address. As a consequence, there may be some repetition between the introduction, materials and methods, results and discussion sections in each chapter.

CHAPTER 1: GENERAL INTRODUCTION

Municipal wastewater effluents (MWWEs) contain complex mixtures of chemicals that are diverse in their structures and biological activities (Reemtsma et al. 1999). There has been increasing concern that some of these chemicals have the potential to disrupt the endocrine system of organisms, which can impact growth, development, and reproduction (Jobling et al. 1998; Hecker et al. 2006; Kidd et al. 2007). These endocrine disrupting chemicals (EDCs) differ from traditional contaminants such as metals and dioxin-like chemicals in that they can be biologically active at very low concentrations, creating unique challenges for traditional risk assessment approaches (Hecker and Hollert 2009). In addition to several well-described EDCs such as natural or synthetic steroid hormones, plasticizers, etc., MWWEs also contain a large number of unknown chemicals that are likely to contribute to environmental endocrine disruption. Therefore, specific screening programs such as the United States Environmental Protection Agency's (US-EPA) Endocrine Disruptor Screening Program (EDSP) have been established to assess the potential hazards that these chemicals may pose to humans and wildlife. Specifically, programs like the EDSP aim to identify chemicals with specific endocrine properties including those that have the potential to interact with the estrogen receptor (ER), androgen receptor (AR) and sex-hormone steroidogenesis.

Traditionally, analytical chemistry has been utilized to identify EDCs in complex mixtures such as MWWEs (Burgess et al. 2013). However, this approach is limited as it does not provide a complete analysis of all biologically active contaminants in a sample, and also does not account for potential interactions of the different compounds present in these mixtures (Samolloff et al. 1983; Ankley, Gerald T. and Mount 1996). Therefore, alternative approaches are needed to better

characterize the overall endocrine disrupting potential of these effluents. One such approach involves the use of targeted *in vitro* bioassays that enable characterization of the specific endocrine activity of complex mixtures since they respond to all chemicals with the same mode of action in a mixture, including unknowns and those for which no analytical detection methods are available (Wilson et al. 2002, 2004; Hecker et al. 2006; Hecker and Giesy 2011). Specifically, this study aimed to apply an *in vitro* bioassay-directed approach using a combination of receptor- and non-receptor-mediated cell-based bioassays as well as targeted chemical analysis to characterize endocrine potentials in influents and effluents of six WWTPs across Canada.

1.1 Endocrine disruption

Endocrine disrupting chemicals are a heterogeneous group of natural and synthetic compounds that can mimic the action of endogenous hormones and/or affect their production, elimination or transport (Sonnenschein and Soto 1998), thus disrupting the homeostatic systems of organisms (Kavlock et al. 1996). Much of the past research on endocrine disruption has focused on the agonistic or antagonistic interaction of chemicals with nuclear hormone receptors, specifically the ER and AR. Disruption of ER- and AR-signaling pathways can result in alterations of several physiological processes in fish, birds, and mammals, including impacts on early embryonic development, growth, and reproductive success or fecundity (Colborn et al. 1993; Janošek et al. 2006). For example, elevated concentrations of estrogens from municipal effluents led to feminization and infertility of male fish in UK rivers resulting in intersex in male fish, characterized by the presence of both male and female gonadal characteristics in the same organism (Jobling et al. 1998). Furthermore, there is increasing evidence to suggest that chemicals that antagonistically bind to steroid hormone receptors, such as anti-estrogenic (e.g. ibuprofen and

naproxen) and anti-androgenic (e.g. carbamazepine, gemfibrozil, DEET) compounds, have the potential to affect the nervous systems of wildlife and humans, as well as the potential to alter behavior and diminish immune system responses (Colborn et al. 1993; Heberer 2002; Stackelberg et al. 2004; Sumpter 2008; Kloas et al. 2009; Zenobio et al. 2014; Ezechiáš et al. 2016; Fraz et al. 2018). Additionally, it has been established that certain chemicals have the ability to inhibit enzymes involved in steroid biosynthesis, resulting in significant reproductive abnormalities in aquatic animals including, but not limited to, decreased testosterone (T) or 17β-estradiol (E2) production (Villeneuve et al. 2007; Ezechiáš et al. 2016; Fraz et al. 2018).

1.2 Types of endocrine disruption

Many EDCs are found at elevated concentrations in MWWEs due to anthropogenic activities (Boxall et al., 2012), and from which they are then released into the aquatic environments with the potential to cause adverse effects in organisms living in downstream environments. Several of these EDCs are relatively new and have very little information available regarding their toxicological properties (Vandenberg et al. 2012). Hence, there is an increasing need for the characterization of the fate and effects of these chemicals to organisms that are at risk of exposure. Most of the research that has been performed to date has focused on exogenous chemicals causing endocrine disrupting effects through binding to the ER, and to a lesser extent to the AR. Disruption of normal estrogen signaling can occur when a compound either agonistically or anatgonistically binds to the ER (Björnström and Sjöberg 2005; Leung et al. 2006). Common chemicals that have been reported to act as estrogens include the natural or synthetic steroid hormones estrone (E1), estradiol (E2), and ethinylestradiol (EE2), as well as several plasticizers and surfactants including bisphenol-A (BPA), octylphenol (OP) and nonylphenol (NP) (Metcalfe et al. 2001; Rodgers-Gray

et al. 2001; Ankley et al. 2003; Kidd et al. 2007; Wright-walters et al. 2007; Sousa et al. 2010a; Jarošová et al. 2014b).

Although there has been a great focus on EDCs that target estrogen-dependent pathways (Sumpter, 2008), equal importance should be placed on chemicals that interact with other pathways of the endocrine system. In particular, chemicals with (anti-)androgenic properties such as DEET and triclosan are of concern, as they are thought to be abundant within aquatic environments and there is increasing research linking the presence of (anti-)androgenic chemicals to some forms of reproductive abnormalities or deficiencies (Johnson et al. 2007; Jobling et al. 2009a; Hill et al. 2010). Therefore, additional research is required to determine the biological significance of (anti-) androgenic compounds, as there is still little information about the distribution and identity of these substances.

Although the ability of a chemical to agonistically or antagonistically bind to sex steroid receptors represents a biological relevant mechanism of endocrine disruption, there are a number of non-receptor mediated process such as the synthesis or elimination of hormones that present an equal concern with regard to endocrine disruption (Sanderson, 2000; Sanderson et al., 2002). Specifically, several studies have found substances in the environment that have the potential to disrupt the production of the natural sex steroid hormones E2 or testosterone (T) (Bláha et al. 2006; Hecker and Hollert 2011). For example, polybrominated diphenyl ethers (PBDEs) used as additive flame retardants were found to disrupt steroidogenesis *in vitro* by decreasing E2 production (He et al. 2008). Furthermore, ibuprofen was found to decrease E2 production *in vitro* and *in vivo* resulting in impaired sperm motility and reproduction of aquatic organisms (Han et al. 2010). Therefore, the combination of receptor-mediated and non-receptor-mediated assays is pertinent

for a comprehensive assessment of the potential endocrine activity of chemicals of concern or complex environmental samples (Grund et al. 2011; Hecker and Hollert 2011; Maletz et al. 2013).

1.2.1 ER receptor-mediated endocrine disruption

Estrogens are responsible for mediating several female reproductive functions including development of feminine sexual characteristics and ovarian maturation, and are primarily synthesized by the gonads; and in most cases, higher amounts are present in females than in males (Wallace 1985; Bondesson et al. 2015). However, according to several studies, human estrogens such as E2, E1, and the synthetic estrogen EE2, which are released through MWWEs into the environment, are responsible for a significant portion of the endocrine disrupting effects seen in aquatic systems (Desbrow et al. 1998; Snyder et al. 2001). Estrogens control the expression of specific genes through the interaction of the ligand-bound ER dimer complex with specific DNA sequences called estrogen-responsive elements (ERE). As a result of this interaction, the expression of specific proteins is regulated by cells that regulate physiological functions (Colborn and Clement, 1992; Colborn et al., 1995). There are two types of ERs; membrane-bound ERs and nuclear ERs, but the majority of related research has focused on nuclear ERs. Once an estrogenic compound (e.g. EE2) binds to the nuclear ER, the ER homodimerizes and interacts with the ERE, which then can trigger the transcription of genes involved in estrogenic response pathways (Figure 1.1) (Gogos et al. 2015). Alternately, membrane ERs, although less researched, also have the ability to elicit an estrogenic response when bound by an estrogenic compound. Pang and Thomas (2010) established that zebrafish oocytes exposed to E2 displayed estrogenic responses through the biding of the membrane ERs. In addition to its natural ligands, the ER is known for binding compounds that have structural similarity to estrogen hormones, which can result in an unwanted

estrogenic response. For example, EE2 is a synthetic estrogen that is present in the vast majority of birth control medication and is excreted by women in an inactive form. However, it can be reactivated during the wastewater treatment process by deconjugation (Peter Guengerich 1990; Larsen et al. 2008). EE2 is considered an EDC as it induces estrogenic responses, and as such can disrupt normal endocrine functions of non-target organisms affecting their reproduction, development, behaviour, and general homeostasis (Nasuhoglu et al. 2012). EE2 is more persistent compared to naturally occurring estrogens (e.g. E2) (Larsen et al. 2008) and has been detected in Canadian wastewater effluents as high as 42 ng/L (Ternes et al. 1999). Adverse effects, such as (de)feminization, (de)masculinization, or intersex in aquatic organisms induced by EE2 at environmentally relevant concentrations have been observed in several studies (Seki et al. 2002; Chikae et al. 2003; Örn et al. 2003, 2006). In addition, exposure to EE2 induces synthesis of vitellogenin (Vtg), a yolk protein precursor that is under strict estrogen control, delay in time to spawn, altered mating behaviour, and a decrease in growth, egg number and fertilization success at environmentally relevant concentrations as low as 0.1 to 10 ng/L (Purdom et al. 1994; Rose et al. 2002; Segner et al. 2003; Nash et al. 2004; Kidd et al. 2007; Larsen et al. 2008; Lange et al. 2009). In addition to EE2 and other estrogenic steroids such as E1, E2, and E3, it has been established that other chemicals such as genistein and BPA can induce estrogenic responses through agonistic binding to the ER (Chikae et al. 2003; Schiller et al. 2013).

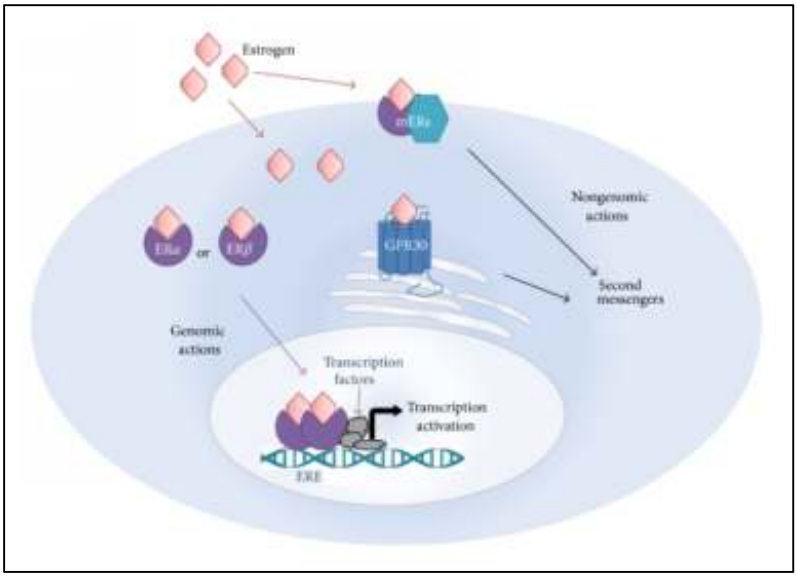

Figure 1.1 – Schematic representation of the cellular response to an ER ligand), such as 17α-ethynylestradiol (EE2). Figure adapted from Gogos et al. (2015). For a detailed description, please see text.

1.2.2 AR receptor-mediated endocrine disruption

The AR is a soluble nuclear receptor protein that predominantly occurs in the cytoplasm. The AR binds androgens such as T, 11-ketotestosterone (11-keto, the active androgen in most fishes) and dihydrotestosterone (DHT, the active androgen in mammals) as well as synthetics androgens (e.g. trenbolone, a cattle growth promotor) to induce a receptor-mediated response mediating male reproduction and anabolic growth promotion. By binding to the AR and inducing a cascade of hormone responses, androgens stimulate and control the development and maintenance of masculine characteristics (Weiss et al. 2009). Androgens play decisive roles in sexual differentiation of the male reproductive tract, accessory reproductive organs, and other tissues during fetal developments (Clark et al. 1998). Binding of androgens such as T and methyl-testosterone to the AR prompts activation of the receptor (AR agonism) resulting in transcription of genes responsible for androgenic effects, similar to the ER receptor (Figure 1.1). Although the AR is regulated mainly by androgens, other hormones or chemicals can affect the process as well by acting as androgen agonists or antagonists. For example, female fish exposed to the anabolic androgen 17β-trenbolone showed a significant increase in masculinization and decrease in Vtg production, as well as increased testicular area and sperm percentage after exposure to 50 ng/L in zebrafish and Japanese medaka (Örn et al. 2006). Additionally, the metabolite of the pesticide dichloro-diphenyl-trichloroethane (DDT), 1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE), was found to act as an androgen, as female amphibians exposed to DDE exhibited occurrence of intersex, thus hindering the sexual delineation and maturation of these organisms (Clark et al. 1998; Boelsterli 2003). Furthermore, environmental pollutants such as pesticides and industrial chemicals, have been found to interfere with the androgen system in both wildlife and humans, mainly by antagonistically binding to the AR (Weiss et al. 2009; Jálová et al. 2013). For example,

anti-androgens such as flutamide, linuron or atrazine have the ability to bind to the AR, but cannot activate the receptor, resulting in AR antagonism (Orton et al. 2009; Weiss et al. 2009; Jálová et al. 2013). To date, only a few studies have focused on compounds with the ability to act as androgen agonists altering endocrine systems, and even less research focusing on the antagonist effects, rendering aquatic environments to potential endocrine risks.

1.2.3 Non-receptor mediated endocrine disruption

There are multiple mechanisms within an organism that regulate the concentrations of circulating sex steroid hormones, and which help to ensure its normal growth, development, and reproduction. Steroidogenesis, or the synthesis of sex steroid hormones, as well as the production of glucocorticoids (cortisol) and mineralocorticoids (aldosterone), is one of these key processes that increases the concentration of steroid hormones when needed (Hecker et al. 2006). Another important factor regulating the availability of hormones are steroid-binding proteins that act as transporter proteins that carry sex steroids throughout the body (Burton and Westphal, 1972). While bound to the protein the sex steroid is inactive, rendering it unable to interact with a target receptor. In a normal functioning state, steroid binding proteins are responsible for ensuring the protection of sex steroids from excretion and metabolism. Finally, eliminatory metabolic processes represent an important regulatory mechanism to maintain endocrine homeostasis as they ensure removal of hormones after they exerted their specific function. When the process is performing properly, the interplay among synthesis, transport as well as metabolism and excretion of steroid hormones are balanced to ensure proper hormone concentrations at target tissues. However, some chemicals have been shown to affect steroidogenesis, the biosynthesis of steroid hormones (Figure 1.2). For example, exposure to two common pharmaceutical drugs, gemfibrozil, and

carbamazepine have been found to decrease the production of 11-keto in zebrafish, which resulted in the decreased whole body, plasma, and testicular hormone levels, as well as reduced reproductive output (Fraz et al. 2018). Therefore, in addition to chemicals that can agonistically or antagonistically bind to the ER and AR, non-receptor mediated processes can significantly alter endocrine functions, specifically if disruption of the synthesis or metabolism/excretion of steroid hormones occurs.

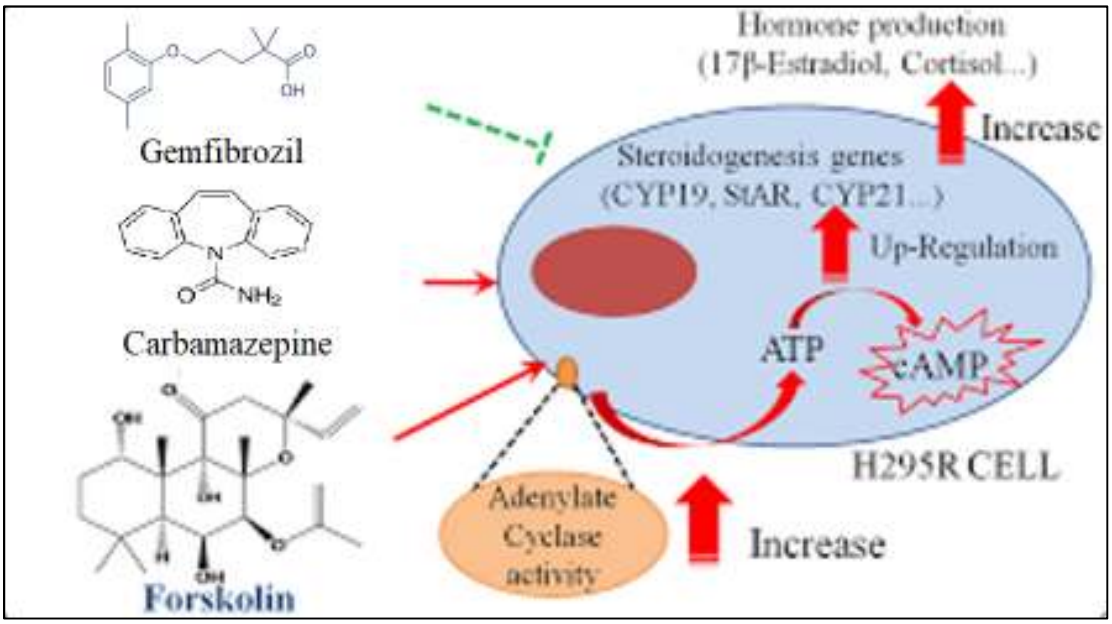

Figure 1.2 – Schematic of hormone production response of H295R cells exposed to chemicals such as gemfibrozil and carbamazepine (adapted from Wang et al. 2015).

1.3 Sources of endocrine disrupting chemicals in aquatic environments

EDCs are introduced into water bodies through a number of different pathways such as runoff from agricultural areas, industrial effluents, and MWWEs discharged into streams, rivers, lakes, and reservoirs (Clara et al. 2005; Benotti et al. 2009). Among the sources of EDCs, MMWEs are considered to be the main contributor to the contamination of aquatic environments with these compounds (Johnson et al. 2007). EDCs found in wastewater include complex mixtures of natural and synthetic hormones (e.g. E2, E1 and EE2) (Ternes et al. 1999; Carballa et al. 2004), plasticizers such as BPA and phthalates, a large number of pharmaceuticals and personal care products (PPCPs), and organic detergents (e.g. nonylphenol) (Metcalf et al. 1973; Jobling et al. 1995; Giesy et al. 2002; Lahnsteiner et al. 2005). MWWEs have been reported to contain compounds with the ability to cause (anti-)estrogenic and (anti-)androgenic effects in aquatic organisms (Purdom et al. 1994).

Traditionally, WWTPs were designed for the removal of organic nutrients, nitrate, and phosphorus; however, removal or breakdown of other contaminants such as PPCPs, industrial chemicals, and detergents is typically incomplete. Studies have demonstrated that some EDCs can be partially removed from wastewater by WWTPs with efficiencies ranging between 60% and 90%, while others pass through without any reduction in concentration (Carballa et al. 2004; Clara et al. 2005; Fent et al. 2006; Díaz-Cruz et al. 2009), rendering receiving aquatic environments at risk from the exposure to EDCs (Halling-Sørensen et al. 1998; Daughton and Ternes 1999; Kümmerer 2001; Zorita et al. 2009). Pharmaceuticals that pass through the human body can be excreted in their native form or as metabolites, and a significant un-metabolized fraction is discarded into municipal wastewater (Ternes 1998a; Falconer et al. 2006; Fent et al. 2006). Because pharmaceuticals are designed with a specific mode of action, they typically have high

potency at low concentrations, stable molecular structure, and are relatively persistent in order to reach their target site in the body before degrading. It is these properties that render PPCPs of particular toxicological concern for aquatic organisms (Fent et al. 2006). Although some PPCPs may be partially broken down during wastewater treatment processes, their continuous release can result in pseudo-persistence in the environment (Daughton and Ternes 1999; Loos et al. 2009). In addition to PPCPs, naturally produced hormones and steroids have been frequently found in surface and groundwater in trace amounts (Barnes et al. 2008). Pharmaceuticals are typically found at greater concentrations in surface waters than groundwater, suggesting that discharge of MWWEs may be the most prominent source of EDCs into the aquatic environment (Barnes et al. 2008).

1.4 Municipal wastewater treatment systems

Traditional wastewater treatment facilities were mainly designed to remove bacteria and organic materials from raw sewage, and thus, many other contaminants including EDCs are not or only partially broken down during the treatment process. As EDCs can be biologically active at very low concentrations, the inability to remove these chemicals raises a significant concern for receiving environments. Several studies have demonstrated that more advanced treatment facilities have the ability to better remove some EDCs of concern from wastewater using activated sludge, ultraviolet (UV) irradiation, ozonation and other advanced treatments (Kirk et al. 2002; Leusch et al. 2005; Rostkowski et al. 2011). According to ERRIS (2013), levels of wastewater treatments vary greatly in Canada, ranging from primary treatments to advanced quaternary treatments (Figures 1.3 to 1.6). In brief, primary treatment systems mainly remove solids, using screening and chemical flocculation to help the breakdown of materials and some chemicals (Figures 1.3 and

1.4). Secondary treatment involves extended aeration, and can also involve an activated sludge treatment step, which replaces some of the chemical flocculation with biological treatment steps comprising digesters with bacteria (Figures 1.3 and 1.5). Tertiary treatments are characterized by even more advanced technologies including biofiltration of nutrients, ammonia, nitrogen, and phosphorus, decreasing the need for the use of chemicals and their discharge into aquatic environments (Figures 1.3 and 1.6). Some facilities also include disinfection systems using chlorine, hypochlorite or UV radiation. Quaternary treatment facilities further enhance the quality of effluent, including potential removal of chemicals such as EDCs, by using ozonation, reverse osmosis, membrane filtration and activated carbon technologies (Figure 1.3) (CCME, 2006). However, due to their high cost, most WWTPs in Canada do not have tertiary or quaternary processes established.

Although there has been a growing number of approaches for the assessment of individual EDCs in context with chemical risk assessment, there is only limited information regarding the effectiveness of current WWTPs technologies to remove EDCs from wastewater. Research that focuses on determining the efficiencies of existing systems will help guide governments and other decision-making bodies in implementing cost-effective solutions for EDC removal.

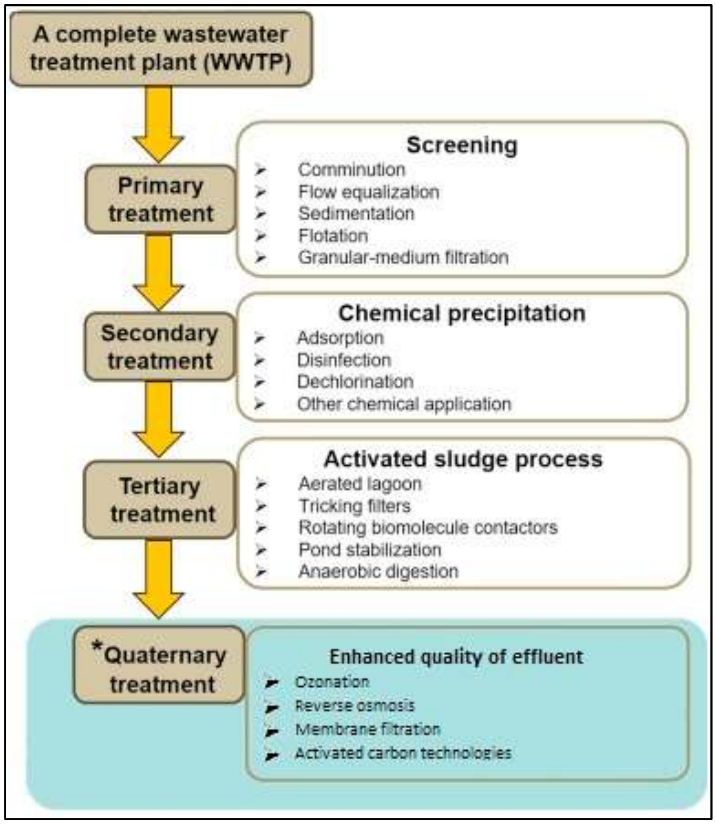

Figure 1.3 – Overview of the different types of treatments used in WWTPs employing primary, secondary, tertiary and quaternary treatments (adapted from (Salama et al. 2017).

1.5 Assessment approaches for endocrine disrupting chemicals

Due to the complex nature of wastewater, one of the major challenges in context with the assessment of the potential toxicological risks of MWWEs is to identify specific compounds that are responsible for biological effects in exposed organisms. Traditionally, analytical chemistry has been utilized to identify chemicals with endocrine disrupting potentials in complex matrices such as effluents (Hecker and Hollert 2009; Hecker and Giesy 2011). However, due to a large number of different types of chemicals present in wastewater, targeted chemical analysis alone is not able to provide a complete and objective exposure assessment. Characterization of exposure using chemical analytical approaches requires prior knowledge of the compounds to be analyzed, and if chemicals were not initially targeted, they will not be identified (Samolloff et al. 1983; Ankley et al. 1996). Therefore, toxicologically relevant compounds could be overlooked by relying solely on the quantification of individual residues using instrumental analyses, underestimating the potential biological risks posed by the effluent. Furthermore, analyzing the vast amount of chemicals in complex environmental mixtures such as MWWEs by chemical analytical methodologies would be extremely time-consuming and prohibitively expensive (Hecker and Giesy 2011). Additionally, when results are obtained through analytical chemistry they are often difficult to evaluate with regard to their potential hazard, as toxicological data of the compounds identified are often not readily available or has not been established yet (Reemtsma et al. 1999). Furthermore, instrumental analyses provide little data on the biological availability and activity of chemicals in mixtures and do not consider the possible interactions among different compounds (e.g. synergism or antagonism) or provide information on their effects. Therefore, over the past decades, a number of alternative approaches to supplement analytical chemistry for the characterization of complex environmental samples have been developed. One of these approaches

is termed Effect Directed Analysis (EDA) and utilizes a combination of biological assays (bioassays), fractionation techniques, and chemical analysis (Brack 2003; Hecker and Hollert 2009).

The bioassay-directed analysis portion of EDA represents the first step to aid in the identification of the biologically active substances in a complex mixture. It is comprised of *in vitro* or *in vivo* mechanistic assays that enable identification of the potential of a sample to interact with specific biological pathways or targets (Hecker and Hollert 2009; Hecker and Giesy 2011). The advantages of *in vitro* bioassays include biological relevance, integrated measures of the combined potency of all chemicals in a complex mixture, and the ability to identify unknown compounds for which analytical methods have not been developed. These assays can also account for interactions between chemicals, which means the effect of two or more chemicals is equal to the sum of the effect of the same chemicals taken separately, which is usually due to the different chemicals acting on the body via the same or a similar mechanism. Many *in vitro* assays can aid in the characterization of the specific mechanism of toxicity a chemical, and in some cases are more sensitive than instrumental analyses. Another advantage of the use of *in vitro* bioassays for initial screening of complex samples such as effluents is that they allow for prioritizing samples based on their biological activity for further analysis, thus avoiding unnecessary and expensive chemical analysis of inactive samples (Jeffcoate 1996; Meager 2006). Most *in vitro* research on EDCs has focused on the ER or the AR, specifically the effects of xenobiotic-mediated binding or the cross-talk of the ER and AR receptors (Villeneuve et al. 1997). However, a number of studies have demonstrated that the disruption of production of steroid hormones or altered expression and activity of steroidogenic enzymes can also occur and that not all effects are the result of direct hormone mimics (Hecker et al. 2006; Grund et al. 2011). Therefore, the use of analytical tools that

only target a few selected modes of action (i.e. ER and AR agonism) is insufficient when dealing with complex matrices such as MWWEs, suggesting that a combination of *in vitro* assays capturing different endpoints and bio-analytical methods may be required (Burgess et al. 2013; Maletz et al. 2013). With regard to the assessment of EDCs, it has been demonstrated that the combination of receptor-mediated and non-receptor mediated assays can be a cost-effective method of evaluating the potential endocrine activity within a complex environmental sample in support to instrumental analysis (Grund et al. 2011).

1.6 *In vitro* vs *in vivo* bioassays

A major issue with the globally increasing need for chemical safety assessment is the reliance of current testing strategies on the use of live animals. Increasing testing demands and requirements result in the need for large numbers of live organisms such as rats, mice, fish, and frogs, creating ethical and economic problems (Jeffcoate 1996; Meager 2006; Hecker et al. 2011). In order to reduce the number of test animals, programs such as US-EPA's EDSP and international organizations such as the Organization for Economic Co-operation and Development (OECD) are increasingly recommending replacement of certain *in vivo* tests with *in vitro* assays that are based on stable cells lines or sub-cellular compartments in their testing schemes (Hecker et al. 2011). *In vitro* tests have a number of advantages over *in vivo* approaches because of their often greater specificity in their response, their amenability to high throughput testing, lower cost, and the fact that they are less time consuming than *in vivo* tests. Most importantly, they address current animal welfare concerns associated with toxicity testing approaches that use a large number of live animals (Gray et al. 1997). Moreover, *in vitro* bioassays include biological relevance and integrated measures of the combined potency of all chemicals in a complex mixture.

Although *in vitro* assays have been shown to be a good tool to support chemical or environmental risk assessment, one has to be aware of their limitations. For example, pharmacokinetics, tissue distribution, and biotransformation of chemicals that occur *in vivo* are often not or only partially accounted for by *in vitro* bioassays. Many cell lines possess only limited metabolic activities, and therefore, substances that undergo metabolic elimination or activation may not be appropriately assessed by these *in vitro* systems. Furthermore, cell-based *in vitro* tests comprise of isolated cells from individual tissues that do not include interactions among different cell types or tissues such as paracrine interactions or endocrine feedback loops, respectively, and which occur in tissue explant or *in vivo* assays (Villeneuve et al. 1997). In addition, *in vitro* bioassays alone do not provide information on the individual compounds causing the measured effects; they only identify candidate groups of causative agents (e.g. those with estrogen or androgen action) in the samples as a first step, and further chemical analysis must be conducted to confirm candidate chemicals.

1.6.1 Bioassay endpoints

The potential of chemicals or environmental samples to interact with the reproductive endocrine system is typically assessed by means of three endpoints: binding to the ER, binding to the AR, and alteration of sex steroid production through interaction with steroidogenic pathways (US-EPA EDSP 2016; OECD 2018). There are a number of *in vitro* assays available for examining different levels of biological complexity, particularly ER binding or transactivation assays (Pons et al. 1990; Legler et al. 1999; Rogers and Denison 2000). Some of the commonly used bioassays engineered to identify and quantify chemicals that can bind to the ER in environmental samples and their ability to complement chemical analysis include the yeast estrogen screen (YES) (Routledge and Sumpter 1996), the ER-CALUX or T47-D (Legler et al. 1999), the MCF-7 (Pons et al. 1990), as

well as the MVLN (Demirpence et al. 1993), and E-SCREEN (Soto et al. 1995) assays. For example, the MVLN assay has been effectively used to determine total (anti-) estrogenicity of complex samples as well as specific potencies of individual estrogens (e.g. E1, E2, estriol (E3) and EE2)(Gutendorf and Westendorf 2001; Snyder et al. 2001). This assay originated from the human MCF-7 breast cancer cell line (Pons et al. 1990), and consists of cells stably transfected with the mouse mammary tumor virus promoter with the firefly luciferase gene under the control of the ER (Demirpence et al. 1993; Gutendorf and Westendorf 2001; Snyder et al. 2001; Coors et al. 2003; Kusk et al. 2011; Demirpence et al. 1993).

Some of the available *in vitro* assays to detect and quantify (anti-)androgenic activities include the yeast androgen screen (YAS) (Sohoni and Sumpter 1998), the AR-CALUX (Van der Burg et al. 2010), and the MDA-kb2 assay (Wilson et al. 2002). The MDA-kb2 assay is one of the most commonly used tests for studying the agonistic or antagonistic interaction of chemicals with the AR. It is a robust and rapid assay, as the cells are generally easy to culture, maintain and results can be consistently replicated (Wilson et al. 2002). This assay has shown to be highly sensitive for the detection and characterization of (anti-) androgenic potentials of chemicals in water samples, such as MWWEs (Wilson et al. 2002). Similar to the MVLN assay, the androgen transactivation assay MDA-kb2 is based on stable transfection of a cell line with two plasmids encoding for the human AR and GR. Specifically, binding of the testosterone- or other AR agonists-AR complex to the androgen response element (ARE) triggers the expression of the reporter gene luciferase, where the bioluminescent response can be measured either in the cellular homogenate or in intact living cells (Wilson et al. 2002).

As previously mentioned, in addition to receptor-mediated mechanisms of endocrine disruption, there are a number of non-receptor mediated processes, such as the modulation of enzymes

involved in the production, transformation, or elimination of steroids, that can also affect the concentrations, availability or actions of hormones, thus also presenting significant concerns regarding endocrine disruption (Sanderson 2000; Ohno et al. 2002; Sanderson et al. 2002; Hilscherova et al. 2004). One assay that has been shown to be useful for the characterization of chemicals or complex environmental mixtures for their potential to disrupt the production of the sex steroids T and E2 is the H295R Steroidogenesis Assay. The H295R assay is based on immortalized human adrenal cancer cells able to produce mineralocorticoids, glucocorticoids, and sex hormones, and integrates effects on all relevant steroid synthesis pathways leading to the production of those hormones (Hilscherova et al. 2004; Zhang et al. 2005; Gracia et al. 2006; Hecker et al. 2007; Gazdar et al. 1990). Endpoints that can be assessed by this assay include the disruption of the production of T, E2 and other steroid hormones either by measuring actual hormone concentrations in cell medium or by assessing changes in activity or expression of steroidogenic enzymes in cells (Sanderson 2000; Zhang et al. 2005; Hecker et al. 2006, 2007, 2011). H295R test methods and measurements have been optimized by several laboratories and standardized by the US-EPA EDSP and OECD (OECD Test No. 456, 2011). Currently, it represents the only validated *in vitro* test for the determination of chemicals or complex mixtures with steroidogenesis disrupting potential (Gazdar et al. 1990; Gracia et al. 2006; Hecker et al. 2006, 2007, 2011).

1.7 Objectives

Little is known regarding the contribution that MWWEs have on the presence of complex mixtures of EDCs in receiving aquatic environments across Canada. To date, few studies have been conducted, predominantly focusing on the Eastern provinces (Metcalfe et al. 2001, 2003; Lishman

et al. 2006; Hicks et al. 2017b, a). In addition, most of the past research has focused on agonists or antagonists of the ER (Jobling et al. 1998; Snyder et al. 2001; Leusch 2008; Leusch et al. 2010; Pessoa et al. 2014). However, chemicals with androgenic and anti-androgenic properties present an equal concern (Ankley et al. 2003; Weiss et al. 2009), as well as chemicals that disrupt other pathways, such as the production of sex steroid hormones, as they also have shown to affect the endocrine systems of organisms (Hecker and Giesy 2011). Furthermore, little is known about the effects of seasonality, particularly under extreme climatic conditions, such as occur in the Canadian prairies, on the efficiency of WWTPs to remove EDCs. Similarly, more information is needed regarding the potential impacts different treatment levels and population demographics can have on the removal of EDCs by WWTPs. Therefore, the overall objective of the research conducted in this thesis was to determine whether MWWEs represent a significant source of EDCs with (anti-)estrogenic, (anti-)androgenic, and steroidogenesis disrupting potencies in Canada. Specifically, endocrine potentials were assessed in influents and effluents collected from six WWTPs in Saskatchewan, Ontario and Quebec, Canada using a battery of *in vitro* assays. Results on *in vitro* bioactivity were then compared with targeted chemical analysis and *in vivo* effects in fish endocrine system (data obtained during two parallel studies) to evaluate the utility of these *in vitro* assays as a predictive tool to prioritize environments at greater risks with the exposure to EDCs. This study was part of a larger project (Aquatic Impact Assessment of Municipal Effluents [AIME]) that aimed to characterize the presence of chemicals with endocrine activities in MWWEs across central and eastern Canada, to assess the potential impacts of these effluents on sensitive wildlife (fish) in receiving environments, and to develop a "toolbox" to efficiently, economically and reliably prioritize MWWEs of concern.

1. *In vitro* characterization of endocrine disrupting potentials of municipal effluents in the Canadian prairies, Saskatchewan.

The first study evaluated two WWTPs in Saskatchewan, Canada, with different treatment levels (Regina, primary lagoon system vs Saskatoon, advanced tertiary system) regarding their efficiency to remove endocrine active compounds from wastewater, and to characterize the potential effects the extreme climatic conditions (hot summers and extreme cold winters with temperatures ranging between -40 to +35°C) may have on this removal efficiency. The specific research objectives were to:

a. Characterize the presence of chemicals in influents and effluents of the Regina and Saskatoon WWTPs that:
    i. agonistically or antagonistically bind to the ER and AR using MVLN cells and MDA-kb2, respectively;
    ii. disrupt the production of the sex steroid hormone E2 using the H295R Steroidogenesis Assay; and
    iii. compare bioassay-derived endocrine potentials to the presence of selected contaminants using targeted chemical analysis
b. Determine whether treatment level and seasonality influence removal efficiency of EDCs by WWTPs;
c. Correlate *in vitro* results with *in vivo* toxicity data for fish endocrine physiology exposed to the same MWWEs either *in situ* or under controlled laboratory conditions as part of two parallel studies to evaluate the predictivity of *in vitro* assays for whole organism responses.

2. Bioassay-directed analysis of endocrine disrupting potencies of municipal effluents in

central and eastern Canada.

The second study was designed to expand on the research under Objective 1 by investigating the endocrine modulating potential in Ontario and Quebec with different treatment levels, population size and climate to generate a more comprehensive understanding of MWWEs as a source of EDCs to aquatic environments across Central and Eastern Canada. Specifically, the objectives of this study were to:

1. Characterize the presence of chemicals in influents and effluents from four WWTPs in Ontario and Quebec that:
    a. agonistically or antagonistically bind to the ER and AR using MVLN cells and MDA-kb2, respectively;
    b. disrupt the production of the sex steroid hormone E2 using the H295R Steroidogenesis Assay; and
    c. compare bioassay-derived endocrine potentials to the presence of selected contaminants using targeted chemical analysis.
2. Determine whether the level of treatment, population size and climate influence removal efficiency of EDCs by WWTPs; and
3. Compare responses to those reported for Saskatchewan WWTPs under Objective 1.

CHAPTER 2: *IN VITRO* CHARACTERIZATION OF ENDOCRINE DISRUPTING POTENTIALS OF MUNICIPAL EFFLUENTS IN THE CANADIAN PRAIRIES, SASKATCHEWAN

PREFACE

Little information regarding the potential effects of municipal wastewater effluents, particularly EDCs, discharged into prairie water bodies is available to date. Therefore, Chapter's 1 aim was to investigate the efficiency of two WWTPs in the prairies, Saskatchewan, Canada to remove EDCs with (anti-)estrogenic, (anti-)androgenicity and steroidogenesis disrupting potentials using a battery of *in vitro* assays, as well as to determine whether treatment level and seasonality influence removal efficiency of EDCs by WWTPs. This chapter was organized as a manuscript for publication in a peer-reviewed scientific journal.

Author contributions:

Tabata Bagatim (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

Sara Hanson (University of Saskatchewan) conducted a parallel study on the endocrine system of fish exposed to municipal effluent downstream of the WWTPs studied, providing information on *in vivo* studies to correlate with *in vitro* results.

Kean Steeves (University of Saskatchewan) conducted a parallel study on the endocrine system of laboratory fish exposed to municipal effluent from the WWTPs studied, providing information on *in vivo* studies to correlate with *in vitro* results.

Hongda Yuan (University of Saskatchewan) helped with the design and performance of experiments to measure selected PPCPs in wastewater using LC-MS.

Steve Wiseman (University of Lethbridge) provided guidance throughout experiments and offered comments and edits to the manuscript.

Natacha Hogan (University of Saskatchewan) provided guidance throughout *in vivo* experiments and offered comments and edits to the manuscript.

Alice Hontela (University of Lethbridge) provided guidance throughout *in vivo* experiments, particularly regarding *in vivo* findings and offered comments and edits to the manuscript.

Paul Jones (University of Saskatchewan) provided guidance throughout chemical analysis experiments, as well as offered comments and edits to the manuscript.

Giesy John (University of Saskatchewan) provided the cell laboratory for the *in vitro* study to be conducted and offered comments and edits to the manuscript.

Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the conception and design of the experiment, offered comments and edits to the manuscript, and provided research funding.

2.1 Abstract

Over the past decades increasing concerns regarding the presence of contaminants in the environment that have the potential to affect the endocrine system of humans and wildlife have been raised. Municipal wastewater effluents (MWWEs) are considered one of the major sources of endocrine disrupting chemicals (EDCs) in surface waters, as conventional wastewater treatment technologies are frequently inefficient at removing these compounds from raw sewage. The aim of this project was to investigate the efficiency of two WWTPs in Saskatchewan, Canada to remove EDCs with (anti-)estrogenic, (anti-)androgenicity and steroidogenesis disrupting potentials using the MVLN, MDAkb2, and H295R Steroidogenesis assays, respectively. The plants investigated were in Regina, a lagoon-based primary treatment system, and in Saskatoon, an advanced tertiary treatment system. The results of this study confirmed generally high efficiencies of WWTPs to remove chemicals with androgenic properties, and low to moderate removal efficiencies of anti-androgens and anti-estrogens. Thus, ER and AR receptor-antagonists remain a significant concern in MWWEs. Furthermore, Regina WWTP with its lagoon-based treatment system was not as efficient at removing contaminants, particularly estrogens, as compared to the more advanced treatment system of the city of Saskatoon. Significant but weak effects on E2 production were observed for a few samples collected from both treatment plants; however, these effects did not follow any clear patterns. Furthermore, seasonality and treatment level seemed to play an important role in the efficiency of EDC removal. Regina effluent is discharged into Wascana Creek, a small surface system with low dilution (<1% during the dry season), and thus, this creek may be particularly at risk with the exposure to EDCs and other emerging contaminants. Results obtained by this *in vitro* study were supported by two parallel studies investigating the effects of the same effluents on fish endocrine physiology, as well as by chemical analysis, demonstrating

in vitro assays can be used as a cost-effective tool for prioritizing endocrine disrupting potential of MWWEs in aquatic environments.

Keywords: emerging contaminants, wastewater, *in vitro*, monitoring

2.2 Introduction

Endocrine disrupting chemicals (EDCs) are a heterogeneous group of natural and synthetic compounds that can impair reproduction, growth, and development of organisms, in some cases at very low concentrations (Kavlock et al. 1996; Jobling et al. 1998; Hecker et al. 2006; Kidd et al. 2007; Hecker and Hollert 2009). EDCs are introduced into water bodies through several different pathways such as runoff from agricultural areas, industrial effluents and municipal wastewater effluents (MWWEs). Among these, MMWEs are considered to be the main contributor to the contamination of surface waters (Clara et al. 2005; Johnson et al. 2007; Benotti et al. 2009). MWWEs contain complex mixtures of a wide variety of known and unknown chemicals including natural and synthetic hormones (e.g. 17β-estradiol [E2], estrone [E1] and ethynylestradiol [EE2]), plasticizers such as bisphenol A (BPA) and phthalates, a large number of pharmaceuticals and personal care products (PPCPs), and organic detergents (e.g. nonylphenol) (Ternes et al. 1999; Carballa et al. 2004; Metcalf et al. 1973; Jobling et al. 1995; Giesy et al. 2002; Lahnsteiner et al. 2005). In addition, they can contain significant quantities of unknown chemicals with the ability to cause (anti-)estrogenic and (anti-)androgenic effects in aquatic organisms (Purdom et al. 1994; Reemtsma et al. 1999).

Traditionally, wastewater treatment plants (WWTPs) were designed to predominantly remove organic materials, nitrate, and phosphorus, and the removal or breakdown of PPCPs, industrial chemicals, and detergents is often incomplete (Hecker and Hollert 2009). Studies have

demonstrated that some EDCs, such as E2 and EE2 can be partially removed from wastewater by WWTPs with efficiencies ranging between 60% and 90%, whiles others bypass without any reduction (Kümmerer 2001; Carballa et al. 2004; Clara et al. 2005; Díaz-Cruz et al. 2009; Zorita et al. 2009). According to Environment and Climate Change Canada (ECCC), levels of wastewater treatments vary greatly across Canada, ranging from less advanced primary treatments to sophisticated quaternary treatments (ERRIS, 2013). While lower level treatment systems tend to be limited in removing xenobiotics from wastewater, quaternary treatment facilities are aimed at enhancing the quality of effluent, including removal of chemicals such as EDCs, by using ozonation, reverse osmosis, membrane filtration and activated carbon technologies (CCME, 2006). However, due to their high cost, most WWTPs in Canada do not have tertiary or quaternary processes (ERRIS, 2013).

The Canadian prairies represent a region of particular concern with regard to release and potential effects of EDCs from WWTPs into surface waters. This geographic region is characterized by an extreme temperature regime and semi-arid climatic conditions. The extreme fluctuations in temperature and precipitation during the various seasons, ranging from positive 35°C to negative 40°C, can significantly impact the efficiency of the biological treatment processes used in WWTPs (Fernandez et al. 2008; Jálová et al. 2013). This, in turn, is likely to result in higher concentrations of nutrients and contaminants, including EDCs, being released into downstream water bodies. Furthermore, dilution of effluents in receiving water bodies can dramatically change among seasons. For example, a study conducted by Waiser et al. (2011b) showed that Wascana Creek, a small water body in the southern Canadian prairies downstream of the city of Regina, SK, can have flows of effluent of up to 99% during the dry season, or during emergency overflow events. However, little information with regard to the potential effects of municipal effluents, and

specifically EDCs, discharged into prairie water bodies is available to date (Waiser et al. 2011a, b). Moreover, the prairie region of Canada has a similar climate compared to parts of Eastern Europe and Asia, with extreme fluctuations in temperatures and semi-arid conditions (Wang and Overland 2004). Thus, improved understanding of effects of such climatic conditions on the removal efficiency of EDCs from municipal wastewater during treatment processes can potentially be used to infer analogous challenges across similar regions in Europe and Asia for which little information on the potential presence and impacts of EDCs is available.

Traditionally, analytical chemistry has been utilized as the primary tool to identify EDCs in complex mixtures such as MWWEs. However, this approach alone is limited as it does not provide a complete analysis of all biologically active contaminants in a sample, including unknown chemicals (Hecker and Giesy 2011). Therefore, *in vitro* bioassays are increasingly used to characterize the specific endocrine activity of complex mixtures as they respond to all chemicals with the same mode of action, including unknowns and those for which analytical detection methods are unavailable (Wilson et al. 2002, 2004, Hecker et al. 2006, 2011). Furthermore, *in vitro* assays can account for interactions among chemicals such as antagonism or synergism (Hecker and Giesy 2011). Although bioassays alone do not provide information on the individual compounds causing the measured effects, they can identify and prioritize candidate groups of causative agents in the samples. Further targeted chemical analysis can then be conducted to pinpoint chemicals or mixture responsible for the effects observed (Villeneuve et al. 1997). Another significant advantage of *in vitro* bioassays is that they enable detection of disruption of specific biological processes without the need for live animal testing. Therefore, programs such as US Environmental Protection Agency Endocrine Disruptor Screening Program (US-EPA's EDSP) and international organizations such as the Organization for Economic Co-operation and

Development (OECD) are increasingly recommending replacement of certain *in vivo* tests with *in vitro* assays in their testing schemes that are based on stable cells lines or sub-cellular compartments (Hecker and Hollert 2011; Hecker et al. 2011; Maletz et al. 2013).

Most studies on EDCs conducted over the past three decades focused on receptor-mediated effects of compounds that bind to the estrogen (ER) or androgen receptor (AR) (Villeneuve et al. 1997; Wilson et al. 2002, 2004). However, recent studies demonstrated that the combination of receptor-mediated and non-receptor-mediated assays was pertinent for a comprehensive assessment of the potential endocrine activity of complex environmental samples such as MWWEs (Grund et al. 2011; Hecker and Hollert 2011; Maletz et al. 2013). In fact, in addition to nuclear receptor-mediated effects, the synthesis of steroid hormones is one of the key processes in endocrine regulation, and which has been shown to be affected by different EDCs including, for example, polychlorinated biphenyls (PCBs) (Li et al. 2004), pesticides (Sanderson et al. 2002) or phthalate esters (Nakajin et al. 2001). In addition, to better understand effects of EDCs, it is important to conduct a combined assessment of agonistic and antagonistic receptor-mediated potential, as the presence of antagonists can decrease agonist responses or have deleterious effects on organisms by inhibiting critical biological processes such as growth or reproduction (Ihara et al. 2014).

Therefore, this study aimed to apply an *in vitro* bioassay-directed analysis approach to determine whether MWWEs represent a significant source of EDCs to aquatic environments in the Canadian prairies. Specifically, the objectives of this study were to 1) characterize the presence of chemicals in influents and effluents of two WWTPs that a) agonistically or antagonistically bind to the ER and AR using MVLN and MDA-kb2 cells, respectively, and/or b) disrupt production of the sex steroid hormone E2 using the H295R Steroidogenesis Assay; 2) determine whether treatment level and seasonality influence removal efficiency of EDCs by WWTPs; 3) correlate *in vitro* results

with fish toxicity data generated by two parallel studies to evaluate the predictivity of *in vitro* assays for organismal responses; and 4) compare bioassay-derived endocrine potentials to the presence of selected contaminants using targeted chemical analysis.

2.3 Methods

2.3.1 Sampling and extraction

Samples of influents and effluents were collected during spring, summer, and early and late winter in 2014 and 2015 from the Regina and Saskatoon WWTPs in Saskatchewan, Canada, representing different effluent types, varying methods of treatment (lagoon-based primary and advanced tertiary, respectively), and a range of climatic conditions over the year (Figure 2.1 and Table S2.1). During each sampling event, two to three 24-hour composite samples were collected at different days spaced over one week per season. Four liters of influent and four liters of effluent were collected in pre-cleaned sterile amber glass bottles from each site per sampling event. Two to three drops of chloroform were added immediately after sampling to each bottle to avoid biological breakdown of compounds and samples were stored in the dark at 4°C to avoid possible biological breakdown prior to further processing. All samples were processed within two weeks of sampling.

Particulates were removed from samples by using glass microfiber filters (0.6µm) recommended for filtration of wastewater (Canadian Life Science, Quebec City, Canada) to prevent obstruction of the extraction cartridges, remove the chloroform previously added, and to permit maximum volumes to be extracted. The used filters were stored at -20°C for further analysis as part of a separate project. Samples were extracted by solid phase extraction (SPE) using Waters Oasis® Hydrophilic-Lipophilic Balance (HLB; 6cc, 150 mg; Mississauga, Ontario, Canada), and Mixed Cation-exchange (MCX; 6cc, 500 mg) cartridges. Each cartridge was conditioned with 5 mL of

methanol followed by 5 mL of ultrapure water. Two liters of the sample or a blank control consisting of 2 L of laboratory ultrapure water were run through each cartridge, at a flow rate of approximately 1 drop/second for extraction. Cartridges were then left to dry, subsequently eluted with 5 mL of ultrapure water followed by 0.1% acetic acid. Dried cartridges were extracted with 5 mL of methanol followed by 5 mL of 1:1 hexane: dichloromethane (DCM). The extracts were then blown down to dryness using a gentle stream of nitrogen, reconstituted in 400 μL of isooctane, and HLB and MCX extracts were combined, resulting in 5000x concentrated samples. Samples were stored at -20^{o}C in 2 mL amber crimp top vials with clear inserts.

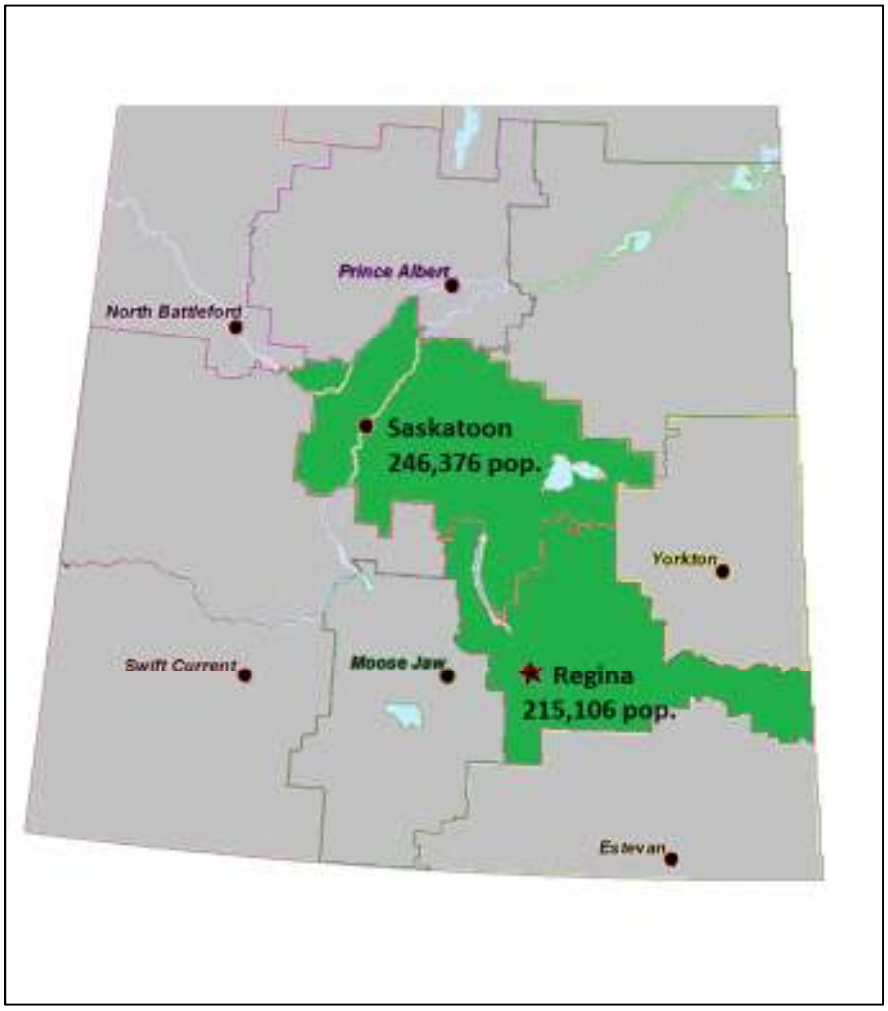

Figure 2.1 – Geographical location of Regina and Saskatoon WWTPs (in green), in Saskatchewan, Canada. Data on population (pop) served are according to Statistics Canada, Census Profile, 2016.

2.3.2 Bioassays

Three cell assays were used to evaluate endocrine disrupting potentials of influents and effluents. MVLN and MDA-kb2 assays were used to determine (anti-)estrogenic and (anti-)androgenic activities, respectively (Demirpence et al. 1993; Wilson et al. 2002). The H295R cell assay was used to measure steroidogenesis disruption (Hecker et al. 2006, 2007, 2011). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA).

2.3.2.1 Cytotoxicity

Prior to conducting endpoint-specific *in vitro* assays (receptor transactivation and steroidogenesis), cytotoxicity of samples to each cell line was assessed using the MTT assay (Mosmann 1983). Cells were seeded at a density in accordance with each cell line assay protocol (see below sections) in 96-well cell culture plates (Corning Inc., NY, USA) with 100 µL of appropriate media for 24 h, as optimized by earlier studies (Ferrari et al. 1990; Ohno and Abe 1991; van de Loosdrecht et al. 1994). Next, the cells were exposed to graded concentrations of extracted samples in isooctane (0.1 % v/v isooctane) in triplicate wells ranging from 0.1x to 10x concentrations of the original samples for 24 h (MVLN, MDA) or 48h (H295R). Samples from two or three collection days per sampling season were analyzed independently. Cell viability was then determined using a spectrophotometer (SpectraMAX 190, Molecular Devices Corporation, California, USA) at an absorbance of 470 nm and 630 nm as described by Mosmann (1983).

2.3.2.2 MVLN cell assay

The MVLN human breast cancer cell line stably transfected with an ER (Demirpence et al. 1993) was used to determine total (anti-)estrogenic activities of the sample extracts. Cells were grown

in maintenance medium Dulbecco's Modified Eagle Medium-F-12 (DMEM-F12) without phenol red (Sigma Aldrich, USA) with 10% fetal bovine serum (FBS; Gibco-Life Technologies Inc., Burlington, Canada) under a 5% CO_2 atmosphere at 37 °C with final pH adjusted to 7.3. Once cells reached approximately 80% confluency, they were trypsinized and seeded into sterile 96-well luminometer plate (Perkin-Elmer, Woodbridge, Canada) at a density of 300,000 cells/mL using 100 µL/well in assay media containing 10% of dextran-charcoal FBS (DCC-FBS; HyClone Laboratories, South Logan, Utah, USA) to reduce concentrations of natural steroids in the bovine serum. Cells were not added to outer perimeter wells, where only PBS was added to be used as background luminescence blanks. After 24 h, cells were exposed to graded concentrations (representing 10x, 3x, 1x, 0.3x and 0.1x concentration of the original samples) of the extracted samples for another 24 h at 37°C as optimized by earlier studies in quadruplicates (wells) (Van Den Belt et al. 2004; Freyberger and Schmuck 2005; Jarošová et al. 2014a). Samples from two or three collection days per sampling season were analyzed independently. An E2 standard curve (1:3 serial dilutions between 0.4-817.1 ng/L E2 in 0.1 % [v/v] Ethanol) and solvent control (SC) (0.1 %v/v Isooctane) were also included on each plate. The medium was then removed, and luciferase activity was measured after adding 75 ml of phosphate buffered saline (PBS) and 75 ml of SteadyLite (Perkin Elmer, USA) with luminescence measured by a microplate luminescence reader (Polarstar Optima, BGM Labtech, Guelph, Canada). Fold-changes relative to the SC were calculated as described by Demirpence et al. (1993), and coefficients of variation (CV) were less than or equal to 20%. Significant activities of samples were confirmed in an independent second experiment. Estrogen equivalents (EEQs) were calculated from the E2 standard curve (four parameters logistic function). EEQs were expressed as ng E2-equivalent/L of influent and effluent samples (1x concentrated). To determine anti-estrogenic activities, the natural ligand E2 was added

to the medium at a concentration that produced a sub-maximal response (1nM E2), and the ability of chemicals to inhibit the luminescence was then determined compared to the anti-estrogen hydroxytamoxifen (HT) added at the same time as described in Demirpence et al. (1993). All other procedures prior to exposure were identical to those described above for the estrogenicity test.

2.3.2.3 MDA-KB2 cell assay

MDA-KB2 human breast cancer cells stably transfected with the AR (Wilson et al., 2002) were used to determine (anti-)androgenic activity. In brief, cells were grown in supplemented medium containing Lebovitz's (L-15) culture media (Gibco) with 10% FBS (Gibco) in a 5% CO_2 atmosphere at 37°C with final pH adjusted to 7.6. Once cells reached approximately 80% confluence, they were trypsinized and seeded into a sterile 96-well luminometer plate (Perkin-Elmer) at a density of 200,000 cells/mL using 100 µL/well. Cells were not added to outer perimeter wells, where only PBS was added to be used as background luminescence blanks. After 24 h, cells were exposed to graded concentrations (10x, 3x, 1x, 0.3x and 0.1x concentration of the original samples) of the extracted samples for 24 h at 37 °C following the protocol by Wilson et al. (2002) for optimal response. A dihydrotestosterone (DHT) standard curve (1:2 serial dilutions between 2.3-145.2 ng/L DHT in 0.1 % v/v isooctane) and solvent control (0.1 % v/v isooctane) were also included on each plate (Figure A2.1). Media was then replaced by 75 ml of PBS and 75 ml of SteadyLite (Perkin Elmer, USA) and luciferase activity was measured using a Polarstar Optima microplate reader (BGM Labtech, Guelph, Canada). Samples were tested in quadruplicates, and CVs were ≤20%. Samples from two or three collection days per sampling season were analyzed independently. Significant activities of samples were confirmed in an independent second experiment. Fold-change relative to the solvent control was calculated as described by Wilson et

al. (2002). Androgen equivalents (AEQs) were calculated from the DHT standard curve (four parameters logistic function). AEQs were expressed as ng DHT-equivalent/L of influent and effluent samples (1x concentrated). To determine anti-androgenic activities, the natural AR ligand DHT was added to the medium at a concentration that produced a sub-maximal response (125 pM DHT) and the ability of chemicals to inhibit the luminescence was then determined compared to the anti-androgen hydroxyflutamide (HF) added at the same time as described in Wilson et al. (2002). All other procedures prior to exposure were identical to those described above for the androgenicity test.

2.3.2.4 H295R cell assay

The H295R human adrenal cancer cell line (Hecker et al. 2006, 2011) was used to determine disruption of steroidogenesis through the modulation of synthesis of E2 in accordance with OECD Test No. 456 (2011). Cells were grown in supplemented medium containing DMEM-F12 Hams (Sigma), 2.5% of BD-serum and 1% of ITS+ premium mix (both from BD Biosciences, Mississauga, Canada) under a 5% CO_2 atmosphere at 37^{o} C with final pH adjusted to 7.4 for a minimum of 4-5 passages to ensure sufficient basal E2 production, and for a maximum of 10 passages as described by Hecker et al. (2006). Once cells reached 80% confluency, they were trypsinized and seeded into 24-well tissue culture plates (Corning Inc., NY, USA) at a density of 300,000 cells/mL using 1000 µL/well. After 24 h, cells were exposed to graded concentrations of the extracted samples (10x, 3x, 1x, 0.3x and 0.1x) for another 48 h, as described by Hecker et al. (2006) for optimal response in triplicate wells at 37 °C. Samples from two or three collection days per sampling season were analyzed independently. Furthermore, forskolin (FOR) (10µM) and prochloraz (PRO) (3 µM) that served as positive controls for induction and inhibition of E2

production, respectively, as well as a SC (0.1 %v/v isooctane/DMSO) and blanks (medium only) were included in each plate. After the exposure, the medium was harvested and concentrations of E2 were determined by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, Anne Arbor, MI, USA) following the manufacturer's instructions. Significant activities of samples were confirmed in an independent second experiment. CVs were ≤20% in all cases.

2.3.3 Chemical analysis

2.3.3.1 Orbitrap chemical analysis - liquid chromatography-mass spectrometry (LC-MS)

Extracts were analyzed using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 µm; 2.1 mm × 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 µl. Ultrapure water (A) and methanol (B) were used as mobile phases. Initially, 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40°C and 10°C, respectively. Data were acquired using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (100 - 1000 m/z) were recorded at resolution R = 70000 (at m/z 200) with a maximum of 3×106 ions collected within 200 ms, based on the predictive automated gain control. SIM scans (m/z = 227.1072, 271.1698, 269.1542, 295.1698) were recorded at a resolution R = 35000 (at m/z 200) with a maximum of 5×104 ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0 m/z. The general mass spectrometry settings

applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375°C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375ºC. Similarly, the settings applied for positive ion mode were: spray voltage, 3.0 kV; capillary temperature, 400ºC; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350ºC.

2.3.3.2 Spike recovery experiments

Prior to chemical analysis, samples were diluted to a 100X concentration in acetonitrile (ACN) and 10 ng/mL of internal standards (Table S2.6) were added for recovery analysis for each sample injection. Extraction efficiencies were determined from a spike recovery experiment. Samples of influent and effluent were extracted following the same SPE method utilized for *in vitro* assays. Non-spiked extracts were first analyzed using the same standards. Pre-existing chemical concentrations in each type of sample were calculated to serve as background. Compound mixtures were then spiked in each sample in triplicates, with concentrations for each standard approximately ten times greater than pre-existing concentrations or maximum allowed concentrations. Recovery was calculated by comparing the detected concentration of each chemical to the expected concentration (Table S2.5). For SIM analysis, estrogenic hormone standards (E1, E2, EE2, and BPA) along with deuterated internal standards (E1, E2, and BPA) were injected at controlled concentrations every six samples. Peak areas of internal standards in samples were compared to peak areas of internal standards alone for recovery calculation. Concentrations of estrogenic hormones were calculated from the peak area ratio of each chemical in a sample and in standards while making reference to the recovery of each individual sample. For full-scan analysis, a standard mixture containing 21 chemicals was injected at 500, 100, 50, 20, 10, 5, 1, 0.1, and 0.01 ng/mL to construct calibration curves. Internal standards containing D3 naproxen, D3 caffeine, and D3 DEET at 10 ng/mL were present in each standard mixture for recovery analysis. The 50

ng/mL standard was also injected after every six samples for concentration calculation. Chemical concentrations in each sample were calculated using the 50 ng/mL standard closest in time as the reference.

2.3.4 Data analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard error of the mean (± 1SEM) and analyzed by one-sample Kolmogorov-Smirnov test for normality, and by Levene's test for homogeneity of variance. Differences among samples (influent vs effluent, WWTPs, sampling day) for normally distributed data were analyzed by analysis of variance (ANOVA), followed by a 2-way Dunnett's test or Tukey's test. Non-parametric data were analyzed by Kruskal Wallis test followed by the Mann Whitney-U test. A probability of $p \leq 0.05$ was considered statistically significant. Dose-response relationships between sample dilutions and magnitude of biological responses were calculated using Microsoft Excel 2016 by fitting data to a four-parameter logistic model. Effective concentrations at which 50% (EC50) and 20% (EC20) of the maximum response for each sample were reported where possible. Androgenic and estrogenic potencies were expressed as the relative change to the SCs, as well as AEQs and EEQs in ng/L based on dilution of a sample as determined by the DHT and E2 standard curves tested simultaneously in each assay plate, respectively. Steroidogenesis disruption was determined by changes in E2 production expressed as relative changes compared to SC.

2.4 Results

2.4.1 Cytotoxicity

Most of the extracted samples, particularly influent, at the greatest concentrations, 10x and 3x, caused significant cell death in all bioassays (MVLN, MDA, and H295R), suggesting one or more compounds in the extracts were acutely cytotoxic (Figures S2.2, S2.3, S2.4). Cytotoxic doses were excluded from further analyses of specific endocrine potentials and 1x concentrations of extracts were used as the highest non-cytotoxic concentration for comparison reasons.

2.4.2 Estrogenicity and anti-estrogenicity

Significant estrogenic potentials relative to the solvent controls were only detected in effluent samples collected from the Regina WWTP during spring and summer with a 2.3-fold (0.9±0.2 ng EEQ/L) and 2.0-fold (0.7±0.1 ng EEQ/L) increase, respectively (Figure 2.2A). No significant increase in estrogenic potentials relative to SCs was observed either in influents or effluents from the Saskatoon WWTP (Figure 2.2C). A significant reduction in estrogenic responses occurred in influent samples during spring; however, such a response was not detected in any other case (Figure 2.2C).

Significant anti-estrogenic potentials relative to the controls were detected in influent and effluent samples from both Regina and Saskatoon during spring and summer (Figure 2.2B and D). During spring, a 0.7- and 0.5-fold change was observed in influent and effluent samples collected from the Regina WWTP samples, and a 0.6- and 0.7-fold change occurred during summer, respectively. In Saskatoon, a 0.4- and 0.5-fold change was shown for influent and effluent samples, respectively, collected during spring, while a 0.6- and 0.7-fold change was measured during summer for

influents and effluents, respectively. No significant anti-estrogenic potentials were detected during early and late winter from either WWTP (Figure 2.2 B and D).

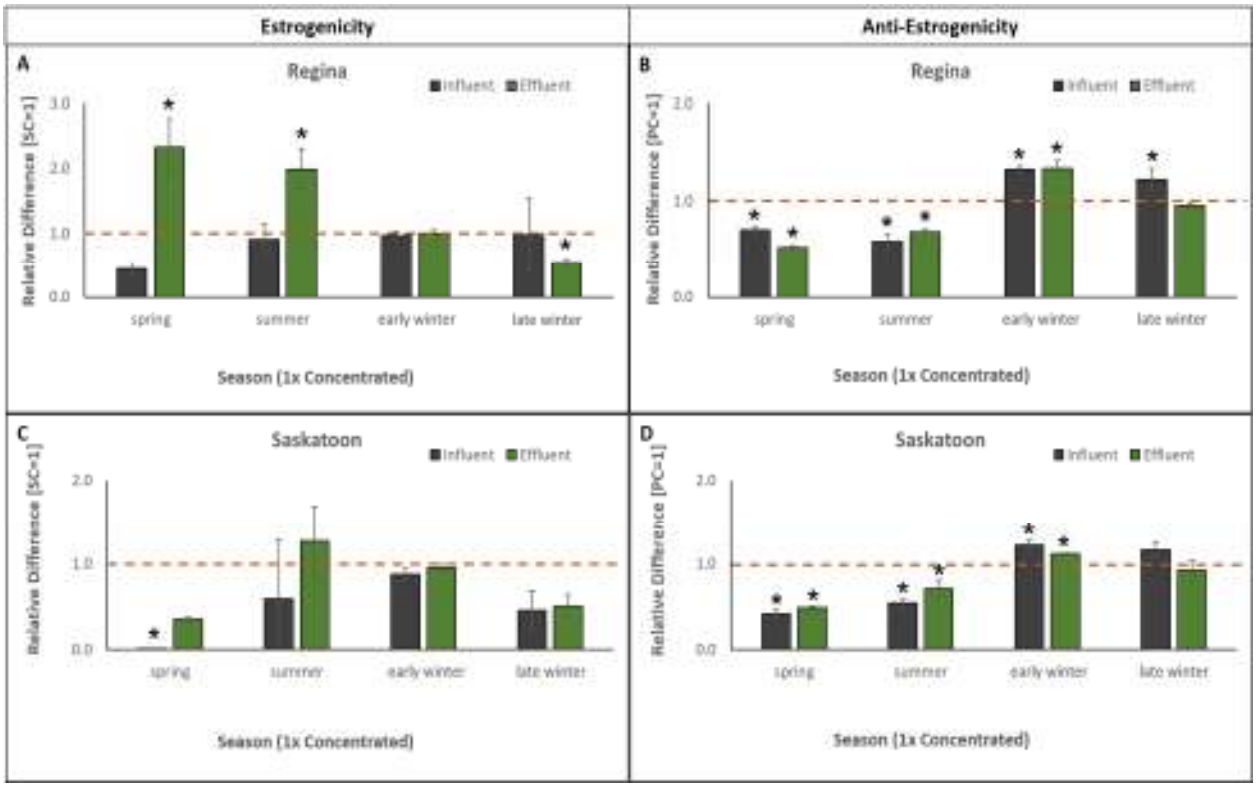

Figure 2.2 - Estrogenic (A and C) and anti-estrogenic (B and D) activity of extracts of influents and effluents (1x concentrated) collected from Regina (A and B) and Saskatoon (C and D) WWTPs during four seasons in 2014/2015 determined using the MVLN *in vitro* assay. Estrogenicity is expressed as relative changes compared to the solvent control (SC). Anti-estrogenic activity is expressed as fold-change relative to the positive control (PC; 81.7 ng/L E2). Data are represented as the mean ± SEM (n=4, wells). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$).

2.4.3 Androgenicity and anti-androgenicity

Influents collected during all four seasons from both WWTPs showed a significant increase in androgenicity compared to SCs (Figure 2.3A and C). AEQs of influents from Regina were found to be 45.0±6.6, 15.2±0.8, 24.4±6.8 and 18.7±5.6 ng AEQ/L, and Saskatoon influents were 6.8±0.6, 52.2±12.0, 63.3±12.0 and 8.9±1.6 ng AEQ/L during spring, summer, early and late winter, respectively. In contrast, no androgenic potentials were observed in effluents, except for the sample collected from the Regina WWTP during early winter, which had a 1.2-fold increase in androgenicity relative to SCs (2.6 ± 0.3 ng AEQ /L) (Figure 2.3A).

Significant anti-androgenic potentials relative to the controls were only observed in influent and effluent samples collected during spring from Regina and Saskatoon (Figures 2.3B and D). A 0.8- and 0.6-fold change was detected for effluents collected from Regina and Saskatoon, respectively, while a 0.1-fold change occurred for the Saskatoon influent sample at the same sampling time.

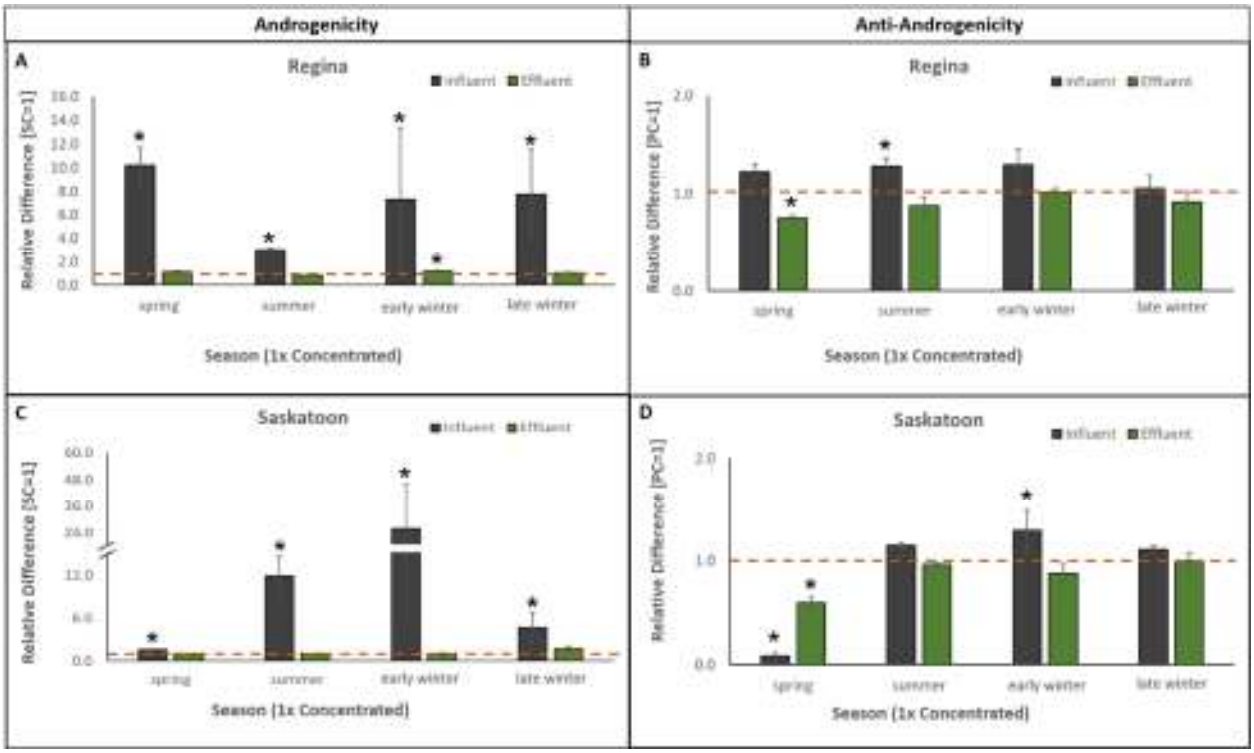

Figure 2.3 - Androgenic (A and C) and anti-androgenic (B and D) activity of extracts of influents and effluents (1x concentrated) collected from Regina (A and B) and Saskatoon (C and D) WWTPs during four seasons in 2014/2015 determined using the MDA-kb2 *in vitro* assay. Androgenicity is expressed as relative changes compared to the solvent control (SC). Anti-androgenic activity is expressed as fold-change relative to the positive control (PC; 36.3 ng/L DHT). Data are represented as the mean ± SEM (n=4, wells). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$).

2.4.4 Steroidogenesis disruption

Analyses of samples with the H295R Steroidogenesis Assay revealed some effects on the production of E2 during select seasons; however, the effects observed did not follow a consistent pattern (Figure 2.4). Specifically, effluent samples from Regina showed a significant increase in the production of E2 during spring and late winter, with a 1.4- and 1.2-fold change compared to the SCs, respectively (Figure 2.4A). Exposure to influent samples from the Saskatoon WWTP caused a 2.3- and 1.5-fold increase in E2-production during spring and summer, respectively, while effluent samples collected during late winter showed a 1.2-fold increase compared to SCs (Figure 2.4B). In contrast, a significant decrease in E2 production was observed in the Regina effluent sample collected during summer and in the Saskatoon effluent sample collected during spring. None of the samples collected during early winter caused any significant effects on E2 production (Figure 2.4).

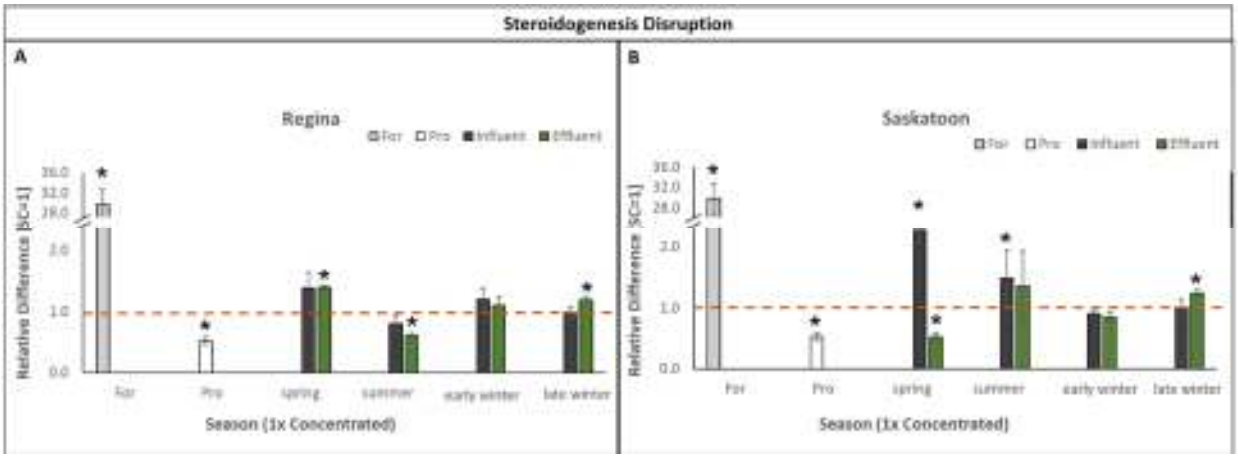

Figure 2.4 - Changes in 17β-estradiol production caused by extracts of influents and effluents (1x concentrated) from Regina (A) and Saskatoon (B) WWTPs sampled during four seasons in 2014/2015 determined by H295R Steroidogenesis Assay. Data are relative changes compared to the solvent control (SC). Results are represented as the mean ± SEM (n=3, wells). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC ($p<0.05$). FOR forskolin [10 μM]. PRO prochloraz [3 μM]. *$p<0.05$.

2.4.5 Chemical analysis

LC-Q Exactive UHPLC analysis revealed the presence of a wide variety of prescription and non-prescription drugs, insecticides, herbicides, and other emergent contaminants in untreated and treated wastewaters (Table S2.2). Most chemicals were detected at greater concentrations in influents compared to effluents, excepted for clofibrate. Among those, contaminants detected at greatest concentrations in influents and effluents during both seasons were carbamazepine (anticonvulsant) at 0.7-17.6 ng/L, clofibrate (antihyperlipidemic) at 30.9-52.7 ng/L, N,N-Diethyl-meta-toluamide (DEET) (insecticide) at 10.3-7,815 ng/L and triclosan (antimicrobial disinfectant) at 0.2-140.7 ng/L. Furthermore, gemfibrozil (antihyperlipidemic) was measured at 268 ng/L only in influent samples from Regina during summer. Also, progesterone (reproductive hormone) occurred at concentrations between 1.8 and 2.3 ng/L but was only detected in influent samples collected from both WWTPs. Other compounds such as reproductive hormones, E2, EE2, E1, Estriol (E3) and Testosterone (T) were below the detection limit in both influent and effluent samples regardless of season (Table S2.2).

2.5 Discussion

In vitro bioassay-based analyses revealed significant endocrine potentials in influents and effluents collected from two WWTPs in Saskatchewan. Endocrine potentials varied among seasons and between treatment systems, confirming that MWWEs represent significant sources of EDCs to receiving environments in Canada.

2.5.1 Estrogenicity and anti-estrogenicity

Estrogenic potentials observed in influents (<MDL-0.7 ng EEQ/L) were less than those detected by other studies conducted in Australia, New Zealand, Czech Republic, Israel and Germany with EEQs ranging between <4.0-185 ng/L (Leusch et al. 2006; Jálová et al. 2013; Shore and Shemesh 2016). However, EEQs detected in effluent samples (<MDL-0.9 ng/L) were similar to those reported by some of these other authors, ranging between <1.0-5.1 ng/L (Leusch et al. 2006; Jálová et al. 2013). In addition, fold-change increases observed for effluents in the present study (<0.5-2.3-fold change), were similar to those observed by Maletz et al. (2013), 1.5-2.9 and 2.0-3.8-fold change in effluent samples using the LYES and ER CALUX® assay, respectively.

The lack of significant estrogenic potentials in influent samples is likely the result of high concentrations of complex mixtures of contaminants, including compounds with anti-estrogenic or general toxic potentials, masking the estrogenic responses of the cell system used. A similar observation was also made by Jálová et al. (2013). Orton et al. (2009) and Jálová et al. (2013) suggested that the presence of chemicals with anti-estrogenic potencies, including pesticides such as linuron or atrazine, could be the cause of reduced or lack of estrogenic responses. Furthermore, effluent samples collected from the lagoon-based Regina WWTP showed significant estrogenic potentials during spring and summer. This is in accordance with earlier reports of lagoon-based WWTPs having low removal efficiencies for estrogenic hormones, ranging between 54 and 80% (Pessoa et al. 2014). In addition, the greater estrogenic response of effluents compared to influents could indicate that during the treatment process some of the anti-estrogen substances that were competing for the same receptor were removed.

Our original hypothesis was that the extreme cold temperatures during winter, particularly in the open lagoon-based Regina WWTP, would result in reduced biological activity, and thus, reduced

elimination of biologically active substances in effluent samples. However, the opposite was the case for estrogenic responses, where estrogenic activity was the highest during summer when significantly greater temperature occurred. Pessoa et al. (2014) hypothesized that the higher estrogenic response in effluents was a result of high evaporation in open lagoon-based systems due to the high temperatures in Brazil, resulting in a greater concentration of compounds with estrogenic properties. This is also in accordance with findings by Fernandez et al. (2008) and Jálová et al. (2013) who reported lower EEQ concentrations in effluents during fall and winter compared to summer in Canada and the Czech Republic, indicating lower temperatures during winter did not negatively influence the removal of estrogenic potency by the WWTPs. Fernandez et al. (2008) attributed this to the fact that the mean daily air temperature varied by as much as 43°C during the sampling period (September/2006 to January/2007), while the effluent wastewater temperature varied only by 7°C, and that the effluent temperature is a more relevant condition influencing estrogens concentrations. Similar variation in temperature was also noticed in the current study, where the mean daily air temperature differences across seasons were 38.8°C in Regina and 36.8°C in Saskatoon (April/2014 to April/2015), while the effluent temperature differences were 19.3°C and 8.2°C in Regina and Saskatoon, respectively. It could be argued that, as Regina showed a higher fluctuation of temperature compared to Saskatoon, the high estrogenicity found in the effluent samples during the spring could potentially be explained by the low temperature of the effluent (2°C) with reduced biological activity; however, this cannot explain the high estrogenicity detected during summer. Similarly, other studies such as Tixier et al. (2003) also could not determine a distinct seasonal pattern in contaminant removal by WWTPs. In conclusion, the original hypothesis that extremely low temperatures will reduce the efficiency of biological degradation activities in WWTPs was not supported by the data obtained during this

study; however, the potential effect evaporation in open lagoon-based systems may have on the concentration of contaminants in effluent warrants further study.

Significant anti-estrogenic potentials were also observed during spring and summer in both influent and effluent samples collected from both WWTPs. This would suggest that the estrogenic signal in effluents from the Regina WWTP may have been suppressed by the co-occurrence of anti-estrogenic compounds. This hypothesis is supported by the remaining significant estrogenicity even in the presence of anti-estrogenic potentials. Jálová et al. (2013) also reported anti-estrogenic potency in extracts from eight sites studied in the Czech Republic, including influents and effluents, as well as rivers downstream of WWTPs, with greatest anti-estrogenicity occurring in samples exposed to WWTP effluent.

As discussed above, Regina wastewater effluent is released into a small stream, Wascana Creek, providing a low dilution during low flow regimes of sometimes less than 1% (Waiser et al. 2011b). This raises significant concerns regarding the possible impacts of contaminants from wastewater to aquatic wildlife downstream of the WWTP. This is particularly true during the spring and summer seasons when greatest EEQs were observed, and which coincide with gonadal maturation and reproductive season of resident fishes such as FHM. EEQs detected in Regina effluent during spring and summer, 0.9 and 0.7 ng/L, were similar to concentrations found by Zha et al. (2008) and Parrott and Blunt (2005) to cause significant adverse effects. Zha et al. (2008) reported that concentrations as low as 0.2 ng/L were able to completely inhibit the reproduction of the F1 generation of Chinese rare minnows (*Gobiocypris rarus*) in a multigeneration study, and Parrott and Blunt (2005) observed significant decreases in egg fertilization and sex ratio alteration (with higher incidence towards females) in FHM at concentrations of EE2 as low as 0.32 ng/L. Nevertheless, the estimates performed in the current studies were more conservative, as they were

based on E2, which are known to be slightly less potent in fish than EE2 compared to these previous studies. In a parallel study that investigated the potential impacts of effluents from the Regina WWTP on the endocrine system of resident fish populations of FHMs, *in situ* and under controlled laboratory conditions Steeves et al. (in preparation) and Hanson et al. (in preparation a,b), no significant effects that could be linked to exposure with estrogens were observed (i.e. induction of vitellogenin production in males, feminization of males or incidence of intersex), and which is in accordance with the low EEQs reported by this study that were below biological effects levels reported by some earlier studies (Segner et al. 2003; Kidd et al. 2007). However, the fish studies found significant impacts on gonadal maturation, decrease in egg production and increase in oocyte atresia in the ovaries in females, which appears to be in accordance with the predominant anti-estrogenic potentials observed.

2.5.2 Androgenicity and anti-androgenicity

Concentrations of androgens reported as AEQs and removal efficiencies of androgenic potentials observed in the present study were similar to those reported by a number of previous studies with concentrations ranging between <MDL and 320 ng AEQ/L in influents, and between <MDL and 163 ng AEQ/L in effluents (Svenson and Allard 2004; Jálová et al. 2013; Shore and Shemesh 2016) compared to 6.8-63.3 ng AEQ/L and <MDL-2.6 ng AEQ/L in influents and effluents, respectively, collected from the Regina and Saskatoon WWTPs. However, Leusch et al. (2005) detected much greater androgenic potencies ranging from 1,920 to 9,330 ng AEQ/L in influents and <6.5 to 736 ng AEQ/L in final effluents. In contrast, other studies reported androgenic potentials in effluents that were consistently below the detection limit (Kirk et al. 2002; Blankvoort et al. 2005; Leusch et al. 2006; Sousa et al. 2010b; Jálová et al. 2013). Androgenic activities in

influent samples observed in the current study were on average 10- to 65-fold greater than estrogenic activities, similar to what was observed by (Leusch et al. 2005) with average differences ranging between 50- to 100-fold. According to Kirk et al. (2002), androgen levels in humans are generally higher than estrogen levels, and most of the androgenic activity in municipal effluents with a high domestic to industrial input ratio is likely caused by androgens excreted by humans. Testosterone levels in plasma of adult human's can be between 3,000 and 10,000 ng/L and between 200 and 750 ng/L in men and women, respectively, while E2 concentrations are normally detected at concentrations between 10 and 60 ng/L in men and between 30 and 400 ng/L in women (Tietz 1976). Therefore, concentrations of androgens in wastewater would be expected to be higher than those of estrogens. Interestingly, neither Testosterone nor other androgenic steroids were measured in influents by LC/MS analyses. However, we did not analyze metabolites, and which may have been contributors to the observed androgenic activities of influent samples.

The relatively low androgenic potentials observed in Regina and Saskatoon effluents indicated high removal efficiencies of androgenic compounds of >83% and >72% by the respective WWTPs, which were in accordance with (albeit slightly lower) removal efficiencies (between 93 and 99%) observed previously (Kirk et al. 2002; Leusch et al. 2005; Jálová et al. 2013). Most of the treatment facilities investigated in the above studies had secondary or greater treatment systems, with the highest removal efficiencies by systems using activated sludge. While the treatment facilities that used primary systems without activated sludge had the lowest removal efficiency (7% during spring in the UK; Kirk et al. 2002). Additionally, most treatment facilities were located within areas with moderate to warm climates (UK, Australia, and the Czech Republic), with air temperature rarely falling below 0°C. Although high removal efficiencies were seen for both Regina and Saskatoon treatments regardless of season, Regina effluent samples

during early winter showed a slight but significant increase in androgenicity (1.2-fold change) compared to controls, which could potentially be due to both temperature and treatment level. In addition to the less advanced technology provided by the Regina WWTP, which does not include an activated sludge treatment. Exposure to average winter air temperatures of -12.4°C for extended periods of time due to longer retention time in the open lagoon systems, can result in significant decrease in biological treatment efficiency as a result of reduced microbial activity (ECCC 2018). As presented in the current study, advanced tertiary treatments, such as used by the Saskatoon plant, have the ability to keep the effluent temperatures more consistent throughout the year, ranging between 9.3 and 12.5^{o}C during early winter to spring, and higher in the summer, while in Regina the effluent temperatures, except for summer, ranged between 2.7 and 4.3C (Appendix Table A2.3). Kirk et al. (2002) also observed lower androgenic and estrogenic activities in effluents of WWTPs with more advanced treatments and reported that the major reduction in activity, due to biological degradation, occurred during secondary treatment, particularly activated sludge. As Regina WWTP is considered a lagoon-based primary treatment, it was able to remove some androgenicity, but in colder months, such as early winter, this removal was incomplete. Surprisingly, the same pattern was not observed during late winter and spring when comparable temperatures were observed, and thus, other factors were likely to have contributed to the fluctuations of androgenicity patterns throughout the year. Similarly, Tixier et al. (2003) were also unable to find a clear pattern of seasonality in Switzerland, where some contaminants increased during snow-melt seasons, while others decreased, illustrating that seasonality can strongly affect removal efficiency. Overall, limited information is available regarding seasonal variability of removal efficiency of compounds with androgenic properties from wastewater, and it remains

difficult to establish clear correlations between climatic/weather conditions and removal efficiencies of androgenic compounds.

While both the Regina and Saskatoon treatment plants showed high removal efficiencies of androgenicity, there were significant anti-androgenic potentials in effluents from both WWTPs in spring. Interestingly, this trend was only observed during the spring season. Increased anti-androgenic potencies exhibited by Regina effluents were likely due to a less advanced treatment level compared to Saskatoon. A similar pattern was observed by Rostkowski et al. (2011), where effluents from WWTPs subjected to less advanced treatments showed a higher number of anti-androgens fractions compared with effluents from more advanced treatments including sand filtration or denitrification stages. In contrast, influent samples collected during spring from the Saskatoon WWTP that had low androgenic agonistic activity revealed highly significant antagonistic activity, suggesting that androgenic effects could have been masked by anti-androgenic compounds within the influent samples tested. Also, the treatment plant was not able to completely remove some of the inhibitors with effluents still showing significant inhibition. This could indicate that both androgenic and anti-androgenic compounds were present in influent samples, competing for the same receptors, and that treatment may have predominantly removed androgenic compounds, resulting in remaining anti-androgenic substances in the effluents. The Saskatoon WWTP showed higher removal efficiency of antagonists (56%) compared to Regina with negative or no removal, suggesting that the advanced Saskatoon treatment system was more efficient at removing AR antagonists from raw wastewater. Similar anti-androgenic potencies to those in both the Regina and Saskatoon WWTPs influent and effluents were observed by Jálová et al. (2013), where most extracts of influents and effluents showed anti-androgenic activities. However, in contrast to what was found in the current study, Jálová et al. (2013) verified that anti-

androgenic potencies were approximately 60% greater in influents than that in effluents, which could be due to different composition of the influent with mixed industrial and domestic (50:50) wastewaters resulting in greater inhibition, compared to prevalent domestic influents in Regina and Saskatoon. In addition, the lesser inhibition detected in effluents investigated in the Jálová et al. (2013) study was likely due to the advanced secondary/tertiary treatment system with four times greater flow capacity than Regina and Saskatoon.

Overall, significant anti-androgenic properties were prevalent in effluents, and thus, may pose a risk to aquatic wildlife. This may be of particular concern for Wascana Creek downstream of the Regina WWTP, due to the low dilution of effluents. In fact, up to 99% of Wascana Creek flows can be composed of effluents during dry seasons (Waiser et al. 2011b, a). To date, androgenic and anti-androgenic effects in wildlife have not been reported as frequently as estrogenic effects; however, some studies verified that chemicals with anti-androgenic properties, such as triclosan and methyl-triclosan that are ubiquitous within the aquatic environment (Johnson et al. 2007; Urbatzka et al. 2007; Hill et al. 2010), may be significant contributors to reproductive dysfunction in aquatic wildlife (Jobling et al. 2009a). The potential biological relevance of anti-androgenic properties of effluents is further supported by two parallel studies that investigated androgenic and estrogenic effects of the Regina and Saskatoon WWTPs *in vivo* using fathead minnows (FHM; *Pimephales promelas*), a resident fish species (Steeves et al. in preparation; Hanson et a. in preparation a,b). Fish exposed to the same effluents in the laboratory or wild fish collected downstream of the Regina WWTP showed no signs of androgenicity such as masculinization of female fish. However, these studies revealed significant anti-androgenic responses, such as delayed gonadal maturation and demasculinization of males, indicating AR antagonists present in

effluents could potentially have contributed to the impacts on normal reproductive functioning of male fish downstream of the Regina WWTP.

2.5.3 Steroidogenesis disruption

Only very few of the samples from either treatment plant caused a significant increase or decrease in E2 production. The magnitude of the effects observed in the present study (<2.0-fold induction of E2) were comparable to those reported by Maletz et al. (2013) after exposure to MWWEs in Germany, who emphasized that fold-changes lesser than 2.0 should not be considered highly relevant effects. However, influent samples from Saskatoon during spring showed a 2.3-fold increase in E2 production, suggesting these samples contained compounds with the ability to stimulate E2 production. In contrast, a significant decrease of E2 production was observed in effluent samples collected from the Regina WWTP during summer and Saskatoon during spring with 0.6- and 0.5-fold changes, respectively. Thus, there was no clear pattern in the presence of compounds that disrupt steroidogenesis either as a function of season or treatment level. Contrary to results observed in the current study, Gracia et al. (2008) detected inhibition of E2 production primarily in influent samples from WWTPs in Hong Kong, SAR, China, while most of the effluent samples did not affect E2 production, except for the outdated sewage WWTP in San Wai. Exposure of H295R cells to MWWE of the latter plant resulted in a 4-fold increase in E2 production, and the authors suggested that this was due to the treatment process not being able to effectively remove EDCs with steroidogenesis disrupting properties. Together with the results from this study, these data emphasize that MWWEs are containing complex mixtures of EDCs that are in continuous flux, resulting in varying effects on endocrine endpoints, likely due to a number of factors, such as variation of chemical concentration and composition in raw sewage,

and removal efficiency. Moreover, a study conducted by Hadrup et al. (2013) that investigated the effects of environmentally relevant mixtures of five pesticides with steroidogenesis stimulating and inhibiting properties, found that mixtures of combinations of inhibitors and inducers did not always respond as predicted based on their individual properties, and that one chemical alone is unlikely to drive the effects of the mixture (Heindel et al. 1995; Olmstead and LeBlanc 2005). As effluents and influents of WWTPs contain highly complex mixtures including stimulators and inhibitors of steroidogenic functions as well as cytotoxic components, this may explain the lack of clear response patterns observed in our study, where in some cases influent samples showed significant increase in E2, and effluent extracts showed a significant decrease, while in other situations no significant response was observed, regardless of the treatment level. To date few studies have been conducted that investigated the potential impacts MWWEs can have on steroidogenic functions, and those that report on effects on E2 production showed significant variation both within and among studies, making it challenging to conduct a more detailed assessment of the steroidogenesis disrupting potentials of MWWEs. Despite the variation due to the reasons listed above, clear signs of steroidogenesis effects were observed, showing that the steroidogenesis disruption assay utilized is an important component for EDC screening. In addition, there is a need for future studies that more thoroughly describe the effects of seasonality as well as compare different treatment levels of WWTPs on steroidogenic pathways including experiments that analyze direct effects on steroidogenic enzymes or their gene expression as well as other endpoints within the steroidogenic pathway such as androgen or corticosteroid production.

2.5.4 Chemical analysis

The current study did not detect the presence of reproductive hormones or other chemicals that have been previously reported to act as hormone receptor agonists. The lack of detection of hormones such as EE2 and E2 was not anticipated, in particular for the effluent of the lagoon-based Regina WWTP. However, this observation was comparable to results from other studies (Ternes et al. 1999; Baronti et al. 2000; Huang and Sedlak 2001; Kolpin et al. 2002), which rarely detected EE2 and E2 in WWTP effluents, and if detections occurred they were at low concentrations. Although endogenous and synthetic hormones were not detected, a number of other compounds were found at elevated concentrations. The four most prevalent compounds included carbamazepine, clofibrate, DEET and triclosan in influents and effluents from the Regina and Saskatoon WWTPs.

Carbamazepine, an anticonvulsant has been found in wastewaters at highly variable concentrations, ranging from <MDL to 1,850 ng/L in influents and 33 to 6,300ng/L in effluents (Table S2.4). In the present study, the concentrations of carbamazepine were found to be lower than measured in many previous studies, ranging from 1.7 to 17.6 and 0.7 to 8.8 ng/L in influent and effluent samples, respectively. Fraz et al. (2018) observed that chronic exposure of fish to carbamazepine at 10,000 ng/L showed decreased androgen levels and fish reproduction in laboratory studies. However, given the low concentrations of carbamazepine detected, it is unlikely that this compound has contributed to any of the biological activities observed. **Clofibrate** is an effective antihyperlipidemic agent (Schulman et al. 2002), detected worldwide at concentrations ranging from <MDL to 360 and 37 to 990 ng/L in influents and effluents, respectively (Table S2.4). In the present study, clofibrate was detected between 35.9 and 52.7 ng/L in influent and between 30.9 and 37.8 ng/L in effluent samples, which is consistent with the 37 ng/L observed by

Brun et al. (2006) in effluent samples in Canada. Previous studies have demonstrated that clofibrate can act as an inhibitor of the estrogen receptor at 41,462 µg/L using YES assay (Ezechiáš et al. 2016), and has the ability to inhibit enzymatic activities involved in the synthesis of active androgens at 242,690 µg/L (Fernandes et al. 2011). However, the concentrations detected in the current study were 1,000 to 10,000-fold less than what was described by Fernandes et al. (2011) and Ezechiáš et al. (2016), and therefore, it is unlikely that the presence of clofibrate has contributed to the anti-androgenicity and anti-estrogenicity observed in this study. **DEET**, an all-purpose individual insect repellent, and has been observed in the environment at highly variable concentrations ranging from 10 to 10,000 ng/L in influent and 140 to 2,238 ng/L in effluent samples (Table S2.4). In the present study, comparable concentration ranges of DEET were observed, between 152 to 7,815 and 10.3 to 3,169 ng/L in influent and effluent samples, respectively. Few studies are available that investigated the endocrine disrupting potential of DEET in aquatic vertebrates such as fish, but one study by Zenobio et al. (2014) observed androgen receptor gene downregulation (0.4-fold change) in female FHM after DEET exposure at 600 ng/L. Concentrations of DEET observed in this study were greater than effect levels previously reported, suggesting the anti-androgenic activity measured *in vitro*, as well as the inhibition of reproductive functions in the parallel fish experiments, could be explained by the presence of this chemical alone or in mixture with other compounds. This warrants further studies to better understand DEET's mode of action and possible endocrine effects. **Triclosan** is an antimicrobial known to be structurally similar to estrogenic and androgenic EDCs, containing molecules with two aromatic rings (Veldhoen et al. 2006; Allmyr et al. 2008). Although, high removal efficiency by WWTPs has been reported with >95% (Table S2.4), its high and continuous consumption results in pseudo-persistence and continuing detection in MWWEs (Waiser et al. 2011b, a). Concentrations of

triclosan worldwide range between 10 and 26,800 ng/L in influent and between 10 and 3,100 ng/L in effluent samples (Table S2.4). In the current study, triclosan was measured in influent samples at the lower range of concentrations previously measured, 6.1-140.7 ng/L, and at lesser concentrations, 0.2-29 ng/L, in effluent samples. Recent studies have shown that triclosan has the potential to interact with the endocrine system of vertebrates via disruption of thyroid hormone homeostasis and possibly the reproductive axis, but effects occurred at much greater concentrations between 20,000 and 100,000 ng/L (Foran et al. 2000; Ishibashi et al. 2004; Raut and Angus 2010). Thus, it is unlikely that the presence of triclosan alone could have contributed to the results observed.

Furthermore, in this study, some chemicals were only found in influent samples, such as gemfibrozil (antihyperlipidemic) at 268 ng/L during summer in Regina and progesterone (reproductive hormone) between 1.8 and 2.3 ng/L in both WWTPs. This indicates that the two studied WWTPs were efficient at removing these compounds and that improvements regarding volume capacity to avoid bypass of untreated raw influent during specific times of the year with potentially higher concentrations of these contaminants is important. Most of the chemicals detected in the present study, except for DEET, were found at concentrations previously reported as "nontoxic"; however, these chemicals are occurring in complex mixtures with each other as well as a myriad of other compounds, and little to nothing is known regarding the potential combinatory effects of these mixtures. Many of these chemicals including hormonally active chemicals, personal care products, and pharmaceuticals are developed to stimulate certain therapeutical or other physiological responses in humans, plants, and animals at low concentrations, and as such may pose a greater risk than traditional contaminants (Daughton and Ternes 1999; Jorgensen and Halling-Sorensen 2000). Moreover, the strong variations of chemicals

concentrations among WWTPs and seasons may be explained by a number of factors also observed by other studies, including daily concentration fluctuations due to consumption, environmental regulations, effectiveness of the WWTPs and seasonal conditions affecting WWTP removal efficiency (Petrovic et al. 2009; Jelić et al. 2012; Luo et al. 2014a).

2.6 Conclusion

The results of this study confirmed generally high treatment efficiency of WWTPs for the removal of androgens throughout the year, while low to moderate removal efficiencies occurred for anti-androgens and (anti-)estrogens. Thus, receptor-antagonists for both the ER and AR remain a significant concern in the effluents of the Regina and Saskatoon WWTPs. Furthermore, the Regina WWTP with its lagoon-based treatment was not as efficient at removing other contaminants, particularly estrogens, as Saskatoon with a more advanced treatment system. This is particularly concerning as Regina effluent is discharged into Wascana Creek, a small surface system with low dilution, which can be composed of 99% effluent before it connects with the Qu'Appelle River downstream (Waiser et al. 2011b), which renders this creek and other low-flow systems in semi-arid environments at higher risk to the exposure with EDCs, especially during the dry seasons. Spring appeared to be the most concerning season, showing the greatest effects with regard to EDCs with MWWEs when receptor and non-receptor mediated assays were compared. The results obtained by the *in vitro* study were supported by two parallel *in vivo* studies, as well as by the chemical analysis, demonstrating *in vitro* assays can be a relevant cost-effective tool for prioritizing potential EDC impacts of MWWEs in aquatic environments.

To conclude, both the seasonality and treatment level seemed to play an important role regarding EDC removal (Table 2.1). Future studies should include WWTPs with different population

demographics, treatment levels, and temperature regimes in order to better understand how these endpoints can affect EDC removal. Furthermore, to better pinpoint the potential main drivers of toxicities of MWWEs, other endpoints such as genotoxicity, metabolic toxicity, corticoids, etc. should be included to identify other relevant stressors in addition to EDCs that could explain the alterations observed in the fish studies. Finally, future studies should be conducted at the Regina WWTP, which has recently undergone a major upgrade, to evaluate removal improvements of EDCs.

Table 2.1: Summary of *in vitro* results describing the endpoints and outcomes of potential risks of MWWEs from Regina and Saskatoon, detected by MVLN (anti-)estrogenicity, MDA (anti-)androgenicity, and H295R (steroidogenesis disruption). YES, effluent from both WWTPs showed at least one season of significant activity. SOME, effluent from one of the WWTPs showed significant activity at least during one season. NO, no significant activity >2-fold change from either WWTP.

Are MWWEs a significante source of EDCs?		
Endpoint	**Outcome**	**MWWEs**
ER	SOME	Regina
Anti-ER	YES	Regina/Saskatoon
AR	NO	None
Anti-AR	YES	Regina/Saskatoon
Steroidogenesis disputors	SOME	Regina/Saskatoon WWTPs affected production of E2, with effluents causing decrease of E2 overall. However, no consistent pattern was observed
Does treament level affect EDC removal?	SOME	Regina showed greater endocrine activity overal
Does seasonality affect EDC removal?	SOME	Spring showed greater endocrine activity overal
Can *in vitro* assays be used as a cost-effective tool for prioritizing potential endocrine disrupting impacts of MWWEs	YES	Results from parallel *in vivo* and chemical analysis studies, were in agreement with *in vitro* findings

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CHAPTER 3: BIOASSAY-DIRECTED ANALYSIS OF ENDOCRINE DISRUPTING POTENCIES OF MUNICIPAL EFFLUENTS IN CENTRAL AND EASTERN CANADA

PREFACE

This chapter was developed based on the results from chapter 2 where the efficiency of two WWTPs in Saskatchewan, Canada were investigated for their ability to remove EDCs with (anti-)estrogenic, (anti-)androgenicity and steroidogenesis disrupting potentials, as well as to determine whether treatment level and seasonality influenced removal efficiency. According to chapter 2, both seasonality and treatment level seemed to play an important role regarding EDC removal; however, a clear pattern, particularly as a function of temperature, was not determined. Therefore, Chapter 3 further evaluated four additional WWTPs across Canada with different treatment levels, temperature regimes, and population demographics in order to better understand how these endpoints could affect EDC removal. This chapter was organized as a manuscript for publication in peer-reviewed scientific journals

Author contributions:

Tabata Bagatim (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

Kean Steeves (University of Saskatchewan) conducted a parallel study on endocrine physiology of laboratory fish exposed to municipal effluent from the WWTPs studied, providing information on *in vivo* studies to correlate with *in vitro* results.

Sara Hanson (University of Saskatchewan) conducted a parallel study on the endocrine system fish exposed to municipal effluent downstream of the WWTPs studied, providing information on *in vivo* studies to correlate with *in vitro* results.

Hongda Yuan (University of Saskatchewan) helped with the design and performance of experiments to measure selected PPCPs in wastewater using LC-MS.

Paul Jones (University of Saskatchewan) provided guidance throughout chemical analysis experiments, as well as offered comments and edits to the manuscript.

Leslie Bragg (University of Waterloo) provided samples from Ontario WWTPs, across Canada as well as previous data collected from her team regarding endocrine disrupting potentials from MWWEs.

Hadi Dhiyebi (University of Waterloo) provided samples from Ontario WWTPs, as well as previous data collected from his team regarding endocrine disrupting potentials from MWWEs.

Mark R. Servos (University of Waterloo) provided samples from Ontario WWTPs, as well as previous data collected from his team regarding endocrine disrupting potentials from MWWEs, as well as guidance throughout the experiments, and comments and edits to the manuscript.

Charles Gauthier (Université du Québec) provided samples from Quebec WWTPs, as well as previous data collected from his team regarding endocrine disrupting potentials from MWWEs, as well as guidance throughout the experiments, and comments and edits to the manuscript.

François Gagné (Environment Canada) provided samples from Quebec WWTPs, as well as previous data collected from his team regarding endocrine disrupting potentials from MWWEs, as well as comments and edits to the manuscript.

John Giesy (University of Saskatchewan) provided the cell laboratory for the *in vitro* study to be conducted and offered comments and edits to the manuscript.

Dr. Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the

conception and design of the experiment, offered comments and edits to the manuscript, and provided research funding.

3.1 Abstract

There has been increasing concern with regard to the release of chemicals that can affect the endocrine system of aquatic organisms by municipal wastewater effluents (MWWEs). In particular, there is significant uncertainty regarding the ability and efficiency of wastewater treatment plants (WWTPs) to remove these compounds from raw sewage. This project aimed to investigate the efficiency of four WWTPs in Ontario and Quebec to remove EDCs, and to compare results with those obtained during an earlier study that characterized the endocrine disrupting properties of influents and effluents from two WWTPs in Saskatchewan. The studies used a combination of three *in vitro* bioassays: the MVLN ((anti-)estrogenicity), MDAkb2 ((anti-)androgenicity), and H295R Steroidogenesis (steroidogenesis disruption) assays. Treatment levels of the investigated WWTPs ranged from lagoon-based primary to tertiary with activated sludge and UV filtration. The results of this study confirmed medium to high treatment efficiencies of WWTPs for the removal of androgens and low to moderate removal efficiencies of anti-androgens and (anti)-estrogens. Thus, ER and AR receptor-antagonists remain a significant concern in MWWEs in Canada that requires further research and analysis. Furthermore, population, seasonality and treatment level seemed to play an important role in driving the efficiency of WWTPs to remove EDCs. The Montreal and Quebec City WWTPs, which serve the largest populations among the cities studied, had the greatest androgenic potentials in both influents and effluents. Both Montreal and Regina, which utilized primary open lagoon systems that are exposed to fluctuations in temperature and have longer retention times, showed the least removal efficiency of contaminants, specifically estrogens, as compared to the more advanced treatment systems utilized by the other WWTPs. Chemical analysis confirmed the results of the *in vitro* assays and verified that *in vitro* assays can be a cost-efficient first step when analyzing the potential presences

of EDCs in MWWEs being discharged into surface waters.

Keywords: emerging contaminants, wastewater, *in vitro*, monitoring

3.2 Introduction

Concerns regarding the potential health effects associated with exposure of humans and wildlife to endocrine disrupting chemicals (EDCs) have been increasing over the past decades. EDCs can be found in the form of both natural and synthetic compounds that can alter the development, reproduction and growth of humans and other animals (Kavlock et al. 1996; Jobling et al. 1998; Kidd et al. 2007). Specifically, EDCs can significantly affect key physiological processes of organisms and have been shown to cause intersex (occurrence of both male and female tissues simultaneous in an organism), reduced egg production, abnormal growth of testes and other reproductive issues (Kidd et al. 2007). One of the primary sources of EDCs to surface waters is municipal wastewater effluents (MMWEs), particularly those discharged into water bodies with low flow rates or that originate from wastewater treatment plants (WWTPs) with inefficient or outdated treatment systems. MWWEs represents complex mixtures with a wide variety of known and unknown chemicals with the ability to affect endocrine systems (Purdom et al. 1994; Reemtsma et al. 1999), including natural and synthetic hormones (e.g. 17β-estradiol [E2], estrone [E1] and ethynylestradiol [EE2]), plasticizers such as bisphenol A and phthalates, a large number of pharmaceuticals and personal care products (PPCPs), and organic detergents (e.g. nonylphenol) (Jobling et al. 1995; Ternes et al. 1999; Giesy et al. 2002; Carballa et al. 2004; Lahnsteiner et al. 2005). Traditionally, WWTPs were designed to primarily filter or settle particulates, organic materials, nitrate and phosphorus from the wastewater; however, degradation or removal of contaminants such as EDCs is often incomplete (Hecker and Hollert 2009). Advanced treatment facilities aim to improve effluent quality by further removing some contaminants, such as EDCs,

using ozonation, membrane filtration and activated carbon (ERRIS, 2013). Previous studies have demonstrated that tertiary and quaternary, and sometimes advanced secondary technologies can improve the efficiencies of WWTPs to remove EDCs (Ternes 1998b; Jálová et al. 2013; Pessoa et al. 2014). In Canada, levels of treatment facilities vary greatly, with some small cities having heavily invested in advanced quaternary treatment technologies, such as Swift Current, SK, while some larger cities, such as Montreal, QC, with a population close to 2 million habitants are still relying on systems built in the 1950s and 1960s with simple primary filtration steps, consisting of lagoons (Lishman et al. 2006). However, due to the high cost associated with advanced treatment systems, only a few municipalities are actively incorporating these types of technologies, meaning the gap among treatment levels used by WWTPs across Canada is significant.

In addition to the high variability of treatment technologies across Canada, extreme climates and highly diverse geographic distribution of the population can make it challenging to understand the occurrence of EDCs and its removal by WWTPs. Canada can be characterized into eleven distinct climate areas with a vast difference in temperature and precipitation, varying from +40°C to -40°C throughout the year. For example, the minimum temperature among the six WWTPs evaluated in the current study can vary by 12.1°C degrees in January, while only by 1.2°C degrees in July (Statistics Canada, 2011). Furthermore, the yearly precipitation shows even greater disparity, with Saskatoon and Regina, which are part of the prairie region, having nearly three times lesser precipitation (350-385 mm yearly), compared to the other cities (900-1100 mm yearly) (Statistics Canada, 2011). Additionally, population density across Canada also varies greatly, with Guelph WWTP serving 131,794 habitants; Saskatoon, Regina, and Kitchener serving 215,000 to 246,000, while Quebec City and Montreal serve the largest number of people, at 531,000 and 1.7 million, respectively (Statistics Canada, 2016).

Despite a general understanding that advanced treatment systems may improve removal of contaminants, little is known about the *in situ* efficiency of WWTPs to remove EDCs, and in particular, how changes in temperature and precipitation can affect their degradation or concentration due to evaporation and dilution (Kirk et al. 2002; Pessoa et al. 2014). Furthermore, little is known regarding the effects population density has on the presence of EDCs in MWWEs, particularly when analyzing less advanced treatment facilities that are at capacity of treatment, and that have different sewage retention and processing times (Kirk et al. 2002; Jálová et al. 2013). It is also important to consider dilution and accumulation of contaminants from WWTPs that have combined domestic sewage and stormwater systems, such as Montreal, during heavy rain events and seasonal snowmelt, where untreated sewage can be bypassed and discharged into the receiving waters (Kirk et al. 2002). Therefore, it is important to establish an understanding of the potential impact treatment level, temperature regime and climate, as well as population size, have on the presence and removal efficiency of EDCs by WWTPs. Moreover, the results obtained from the six cities examined within this study can potentially provide information to cities with similar climates and precipitation, specifically in Europe and Asia (Wang and Overland 2004).

To date, traditional chemical analysis has been the most utilized method for identifying the presence of EDCs in complex environmental samples such as municipal effluents (Hecker and Giesy 2011). However, wastewater influents and effluents are complex mixtures of thousands of chemicals, making individual chemical analysis difficult and cost prohibitive at times. In fact, many of the compounds present in these mixtures are unknown and/or do not yet have analytical procedures established that would enable their reliable quantification, and as such chemical analysis cannot completely assess all the biologically active contaminants within a sample (Brack 2003; Hecker and Giesy 2011). Additionally, many of the chemicals present in MWWEs have no

or only limited toxicity data associated with them, making it difficult to predict any potential biological risks (Brack 2003; Hecker and Giesy 2011). Therefore, bioassays, in particular, mechanism-specific *in vitro* assays, are increasingly used in conjunction with targeted chemical analysis of complex environmental samples such as effluents as they can characterize the specific biological activity within a sample. Specifically, bioassays respond to all the chemicals with the same mode of action (e.g. (anti-)estrogenic and (anti-)androgenic compounds) including unidentified ones for which analytical detection is unavailable, even at low concentrations (Wilson et al. 2002, 2004, Hecker et al. 2006, 2011). However, while bioassays can help identify and prioritize candidate groups of causative agents in complex samples, they cannot pinpoint individual chemicals. Subsequently, it is recommended to combine bioassays with targeted chemical analysis to identify the causative chemicals or mixtures responsible for the effects observed (Villeneuve et al. 1997).

Traditionally, studies conducted to characterize the presence of EDCs in MWWEs have focused on compounds binding to the estrogen receptor (ER) or the androgen receptor (AR) (Wilson et al. 2002). However, several studies have demonstrated that chemicals with different endocrine mechanisms of action such as inhibitors or inducers of steroidogenesis can be of equal importance, and thus, efforts are increasingly made to include both receptor and non-receptor mediated assays for the comprehensive assessment of endocrine activity in complex mixtures (Grund et al. 2011; Hecker and Hollert 2011; Maletz et al. 2013). In particular, the H295R Steroidogenesis Assay has been shown to be a useful tool for the detection of compounds that can disrupt production of steroid hormones (Nakajin et al. 2001; Li et al. 2004).

Given this background, this study aimed to use an *in vitro* bioassay-directed analysis approach to determine endocrine disrupting potentials of MWWEs from different WWTPs across central and

eastern Canada, and whether they might pose a potential risk to the receiving aquatic environment. Specifically, the objectives of this study were to: 1) characterize the presence of chemicals in influents and effluents of six WWTPs that a) agonistically or antagonistically bind to the AR and ER using MDA-kb2 and MVLN cells, respectively, and/or b) disrupt the production of the sex steroid hormone E2 using the H295R Steroidogenesis Assay; 2) determine whether level of treatment and climate influence removal efficiency of EDCs by WWTPs; and 3) compare bioassay-derived endocrine potentials to the presence of selected contaminants using targeted chemical analysis

3.3 Methods

3.3.1 Sampling and extraction

Municipal wastewater influent and effluent samples were collected from four WWTPs across Canada in 2014 during the spring and summer months: Guelph and Kitchener, ON, and Quebec City and Montreal, QC. Furthermore, data from chapter 2, investigating two WWTPs in Saskatchewan (Regina and Saskatoon), were included for comparison. The samples were characterized according to their treatment technologies (lagoon-based, primary and advanced tertiary technologies), population size, and climatic conditions throughout the year (Table S3.1 and Figure 3.1). WWTPs were divided into two main categories, low concern (Saskatoon, Guelph, and Quebec City) and high concern (Regina, Kitchener, and Montreal), according to their treatment level (Table S3.1).

Sampling, as well as preservation and extraction of wastewaters, was in accordance with the procedures described in chapter 2. Briefly, each sampling event consisted of 24-hour composite samples collected during two to three days over one week per season, where four liters of influent,

and four liters of effluent were collected and stored separately in pre-cleaned sterile amber glass bottles from each location. Two to three drops of chloroform were added immediately after collection to avoid breakdown of compounds, which were removed by subsequent filtration, and samples were stored in the dark at 4°C prior to analysis, which occurred within two weeks of sampling. Organic chemicals were extracted from 2L of samples or a blank control consisting of 2L laboratory ultrapure water filtered through 0.6μm glass microfiber filters (Canadian Life Science, Quebec City, Canada) by solid phase extraction (SPE) using Waters Oasis® Hydrophilic-Lipophilic Balance (HLB) 6cc, 150 mg, and Mixed Cation-exchange (MCX) 6cc, 500 mg (Mississauga, Ontario, Canada) as described in chapter 2. The hexane/dichloromethane extracts from HLB and MCX cartridges were then blown to near dryness under a gentle stream of nitrogen, and subsequently combined and reconstituted in 400 μL of isooctane. The final concentration of each sample was consequently 5,000X more concentrated than the unprocessed samples. Extracted samples were stored at -20^{o}C in 2 mL amber crimp top vials with clear inserts.

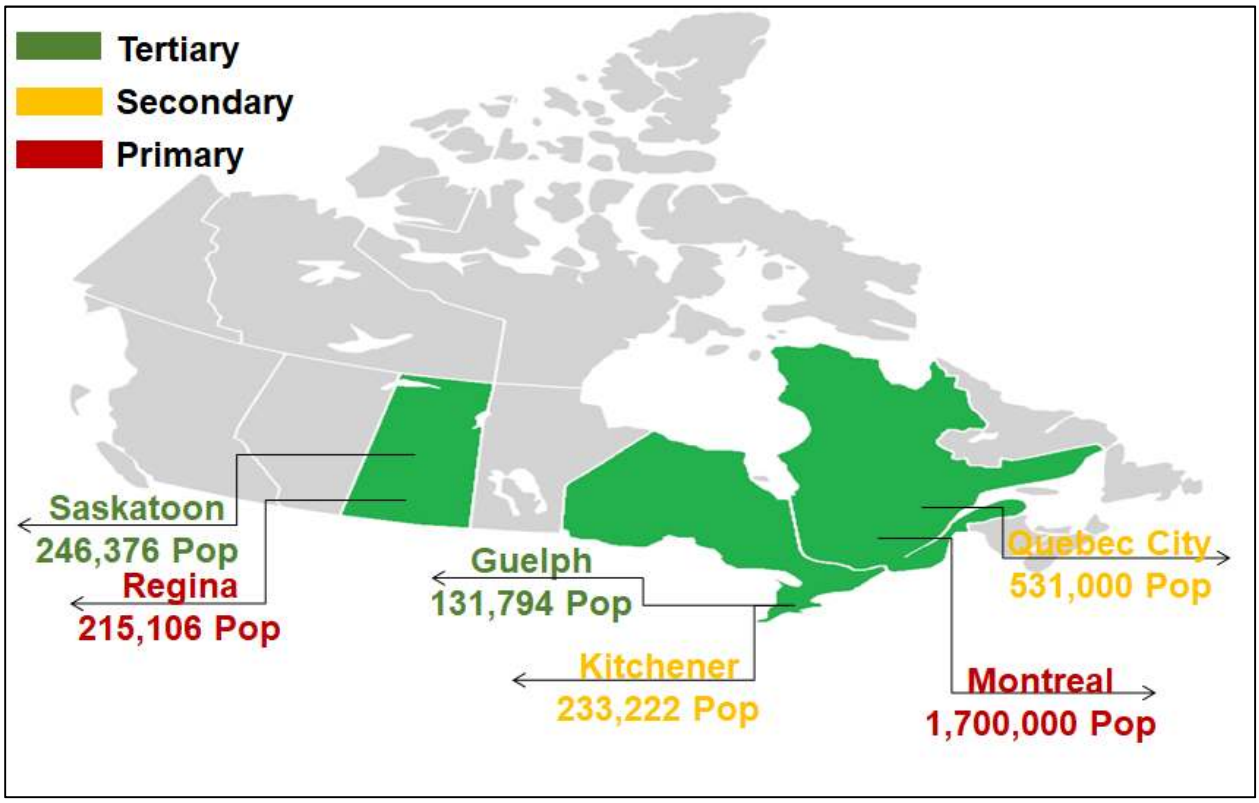

Figure 3.1 – Geographical location (in green), treatment level of the six WWTPs studied across Canada. Data on population (pop) served are according to Statistics Canada, Census Profile, 2016.

3.3.2 Bioassays

MVLN and MDA assays were used to determine (anti-)estrogenic and (anti-)androgenic activities, respectively (Demirpence et al. 1993; Wilson et al. 2002), while the H295R cell assay was used to measure disruption of E2 production (Hecker et al. 2006, 2007, 2011). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA).

3.3.2.1 Cytotoxicity

Each cell line underwent cytotoxicity tests prior to conducting endpoint-specific *in vitro* assays using the MTT assay (Mosmann 1983) (Figures S3.2 to S3.7). Cells were seeded in 96-well cell culture plates (Corning Inc., NY, USA) at a density in accordance with each cell line assay protocol (see below sections) with 100µL of appropriate media for 24 hours as optimized and described by earlier studies (Ferrari et al. 1990; Ohno and Abe 1991; van de Loosdrecht et al. 1994). Subsequently, cells were exposed to graded concentrations of extracted samples in 0.1% isooctane in triplicate wells for 24 h (MVLN, MDA) or 48 h (H295R). Samples from two or three collection days per sampling season were analyzed independently. Concentrations ranged from 0.1x to 10x of the original samples. After exposure, 10 µL of MTT assay dye solution (Biotium, Fremont, USA) was added to each well and incubated for 2-4 hours at room temperature. Last, 200µL of dimethyl sulfoxide (DMSO) was added to each well, and cell viability was measured at an absorbance of 470nm and 630nm using a spectrophotometer.

3.3.2.2 MVLN cell assay

To determine total (anti-)estrogenic activities of the sample extracts from influent and effluent, the MVLN human breast cancer cell line stably transfected with the ER (Demirpence et al. 1993) was

used. Specific procedures followed were in accordance with those previously described in chapter 2 for wastewater extracts. Cells were cultured in maintenance medium Dulbecco's Modified Eagle Medium-F-12 (DMEM-F12) without phenol red (Sigma Aldrich, USA) with 10% FBS (Gibco) under a 5% CO_2 atmosphere at 37 °C with final pH adjusted to 7.3. Cells were seeded at a density of 300,000 cells/mL using 100 µl/well into a sterile 96-well luminometer plate (Perkin-Elmer) in assay media containing 10% of dextran-charcoal FBS (DCC-FBS; HyClone Laboratories, South Logan, Utah, USA) to decrease concentrations of natural steroids in FBS. Cells were not added to outer perimeter wells, where only PBS was added as a background for luminescence blanks. After 24h, cells were exposed to graded concentrations of the extracted samples (10x, 3x, 1x, 0.3x and 0.1x) or a serial dilution of an E2 standard in quadruplicate wells for another 24h as optimized by earlier studies (Van Den Belt et al. 2004; Freyberger and Schmuck 2005; Jarošová et al. 2014a) at 37 °C. Samples from two or three collection days per sampling season were analyzed independently. Final dosing concentrations of E2 standards (1:3 serial dilution) ranging between 0.4-817.1 ng/L in 0.1% ethanol vehicle, as well as SC were included in each plate. The media was then replaced by SteadyLite (Perkin Elmer, USA), and estrogenicity was measured by a microplate luminescence reader (Polarstar Optima). Fold change relative to the SC was calculated as described by (Demirpence et al. 1993) and coefficients of variation (CV) were less than or equal to 20%. Significant activities of samples were confirmed in an independent second experiment. Estrogen equivalents (EEQs) were calculated from the E2 standard curve (four parameter logistic function). EEQs were expressed as ng E2-equivalents/L of influent and effluent samples from the six WWTPs at 1x concentration during spring and summer of 2014. In a separate exposure, to determine anti-estrogenicity, the natural ER ligand E2 was added to the medium at a concentration that produced a sub-maximal response (81.7 ng/L; 1nM E2), while the remaining test procedures

were identical to those described above. The ability of chemicals to inhibit luminescence was then determined compared to the anti-estrogen hydroxytamoxifen (HT) added at the same time as described in Demirpence et al. (1993).

3.3.2.3 MDA-KB2 cell assay

The human breast cancer cell assay stably transfected with an androgen receptor (AR), namely MDA-KB2 (Wilson et al. 2002), was used to determine (anti-)androgenic activities of wastewater extracts as previously described in chapter 2. First, cells were cultivated in supplemented medium containing Lebovitz's (L-15, Gibco-Life Technologies Inc. Burlington, Canada) with 10% Fetal Bovine Serum (FBS) (Gibco) in a 5% CO_2 atmosphere at 37°C with final pH adjusted to 7.6. Cells were seeded at a density of 200,000 cells/mL using 100 µL/well into a sterile 96-well luminometer plate (Perkin-Elmer, Woodbridge, Canada). Cells were not added to outer perimeter wells, where only PBS was added to be used as background luminescence blanks. After 24h, cells were exposed to graded concentrations of the extracted samples (10x, 3x, 1x, 0.3x and 0.1x) or a DHT standard curve in quadruplicate wells for another 24h at 37 °C following the protocol by Wilson et al. (2002) for optimal response. Dosing solutions were prepared by pre-diluting either DHT standards or samples in supplemented media. Final dosing concentrations of DHT standards (1:2 serial dilution) ranging between 2.3-145.2 ng/L in 0.1% isooctane vehicle, as well as solvent control (SC) (0.1 %v/v Isooctane) were included in each plate. After exposure, medium was removed, and androgenic activity was determined by adding SteadyLite (Perkin Elmer, USA) with luminescence measured by microplate luminescence reader (Polarstar Optima, BGM Labtech, Guelph, Canada). Samples from two or three collection days per sampling season were analyzed independently. Significant activities of samples were confirmed in an independent second experiment. Fold

change relative to the SC was calculated as described by Wilson et al. (2002), and androgen Equivalents (AEQs) were calculated from the DHT standard curve (four parameter logistic function). AEQs were expressed as ng DHT-equivalent/L of influent and effluent samples from the six WWTPs at 1x concentration during spring and summer of 2014. In a separate exposure, to determine anti-androgenic activities, the natural AR ligand DHT was added to the medium at a concentration that produced a sub-maximal response (36.3 ng/L; 125pM DHT), while the remaining test procedures were identical to those described above. The ability of chemicals to inhibit luminescence was then determined compared to the anti-androgen hydroxyflutamide (HF) added at the same time as DHT as described in Wilson et al. (2002).

3.3.2.4 H295R cell assay

Steroidogenesis disruption was measured using the H295R human adrenal cancer cell line H295R through the modulation of synthesis of E2 following the OECD Test No. 456 (2011) (Hecker et al. 2006, 2007, 2011), with modifications for wastewater extracts as described in chapter 2. Cells were cultivated in supplemented medium containing DMEM-F12 Hams (Sigma), 2.5% of BD-serum and 1% of ITS+ premium mix (both from BD Biosciences, Mississauga, Canada) under atmosphere at 37ºC and 5% CO_2 with final pH adjusted to 7.4, for a minimum of 4-5 passages to ensure sufficient basal E2 production, and for a maximum of 10 passages as described by Hecker et al. (2006). Once cells reached 80% confluency, they were seeded into 24-well tissue culture plates at a density of 300,000 cells/mL using 1000 µl/well (Corning Inc., NY, USA). After 24h, cells were exposed to graded concentrations of the extracted samples (10x, 3x, 1x, 0.3x and 0.1x) for another 48h as described by Hecker et al. (2006) for optimal response in triplicate wells at 37 °C. Samples from two or three collection days per sampling season were analyzed independently.

Furthermore, forskolin (FOR) (10μM) and prochloraz (PRO) (3 μM), used as positive and negative controls for induction and inhibition of E2 production, respectively, as well as a SC (0.1 %v/v isooctane/DMSO) and blanks (medium only), were included in each plate. Following the exposure, medium was harvested and concentrations of E2 were measured by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer's protocol. Significant activities of samples were confirmed in an independent second experiment. CVs were ≤20% in all cases.

3.3.3 Chemical analysis

3.3.3.1 Orbitrap chemical analysis - liquid chromatography mass spectrometry (LC-MS)

Compounds were identified by reference to chemical standards via accurate mass and ms/ms comparison. Extracts were analyzed using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 μM; 2.1 mm × 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 μL. Ultrapure water (A) and methanol (B) were used as mobile phases. Initially, 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40°C and 10°C, respectively. Data was obtained using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (100 - 1000 m/z) were recorded at resolution R = 70000 (at m/z 200) with a maximum of 3×106 ions collected within 200 ms, based on the predictive automated gain control. SIM scans (m/z =

227.1072, 271.1698, 269.1542, 295.1698) were recorded at a resolution R = 35000 (at m/z 200) with a maximum of 5×104 ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0 m/z. The general mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375°C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375ºC. Similarly, the settings applied for positive ion mode were: spray voltage, 3.0 kV; capillary temperature, 400ºC; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350ºC.

3.3.3.2 Spike recovery experiments

Prior to chemical analysis, samples were diluted to a 100X concentration in acetonitrile (ACN) and 10 ng/mL of internal standards (Table S3.4) were added for recovery analysis for each sample injection. Extraction efficiencies were determined from a spike recovery experiment. Samples of influent and effluent were extracted following the same SPE method utilized for *in vitro* assays. Non-spiked extracts were first analyzed using the same standards. Pre-existing chemical concentrations in each type of sample were calculated to serve as background. Compound mixtures were then spiked in each sample in triplicates, with concentrations for each standard approximately ten times greater than pre-existing concentrations or maximum allowed concentrations. Recovery was calculated by comparing the detected concentration of each chemical to the expected concentration (Table S3.3). For SIM analysis, estrogenic hormone standards (E1, E2, EE2, and BPA) along with deuterated internal standards (E1, E2, and BPA) were injected at controlled concentrations every six samples. Peak areas of internal standards in samples were compared to peak areas of internal standards alone for recovery calculation. Concentrations of estrogenic hormones were calculated from the peak area ratio of each chemical in a sample and in standards

while making reference to the recovery of each individual sample. For full-scan analysis, a standard mixture containing 31 chemicals was injected at 500, 100, 50, 20, 10, 5, 1, 0.1, and 0.01 ng/mL to construct calibration curves. Internal standards containing D3 naproxen, D3 caffeine, and D3 DEET at 10 ng/mL were present in each standard mixture for recovery analysis. The 50 ng/mL standard was also injected after every six samples for concentration calculation. Chemical concentrations in each sample were calculated using the 50 ng/mL standard closest in time as the reference.

3.3.4 Data analysis

Dose-response relationships were calculated by fitting data to four-parameter logistic models using Microsoft Excel 2016. Androgenic and estrogenic potencies were expressed as the relative change to the SCs, as well as AEQs and EEQs in ng/L based on dilution of a sample as determined by the DHT and E2 standard curves tested simultaneously in each assay plate, respectively. Steroidogenesis disruption was determined by changes in E2 production expressed as relative changes compared to SC.

Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard error of the mean (± 1SEM) and analyzed by one-sample Kolmogorov-Smirnov test for normality, and by Levene's test for homogeneity of variance. Differences among samples (influent vs effluent, WWTPs, sampling day) for normally distributed data were analyzed by analysis of variance (ANOVA), followed by a 2-way Dunnett's test or Tukey's test. Non-parametric data were analyzed by Kruskal Wallis test followed by the Mann Whitney-U test. A probability of $p \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Cytotoxicity

Most of the extracted samples, particularly influent, at the highest concentrations (10x and 3x) caused high cell mortality in all bioassays (MVLN, MDA, and H295R), suggesting these extracts contained one or more acutely toxic compounds (Figures S3.2 to S3.7). Cytotoxic concentrations were excluded during further investigations of endocrine disrupting potentials, and the highest non-toxic dose, 1x, was used to compare results among WWTPs.

3.4.2 Estrogenicity and anti-estrogenicity

Most of the samples did not cause significant increases in estrogenic activity. However, samples of influents collected during spring from Montreal caused a 2.9-fold increase (0.9 EEQ ng/L) in estrogenicity, and samples of effluent collected from Regina during both spring and summer caused a 2.3- (0.9 EEQ ng/L) and 2.0-fold (0.7 EEQ ng/L) increase in estrogenicity, respectively (Figures 3.2A and 3.2B). No significant increase in estrogenicity was observed in any of the other samples. In contrast, all samples of both influent and effluent extracts from all six WWTPs showed significant anti-estrogenic activities during both seasons, ranging between 0.4- and 0.7-fold change (Figures 3.2C and 3.2D).

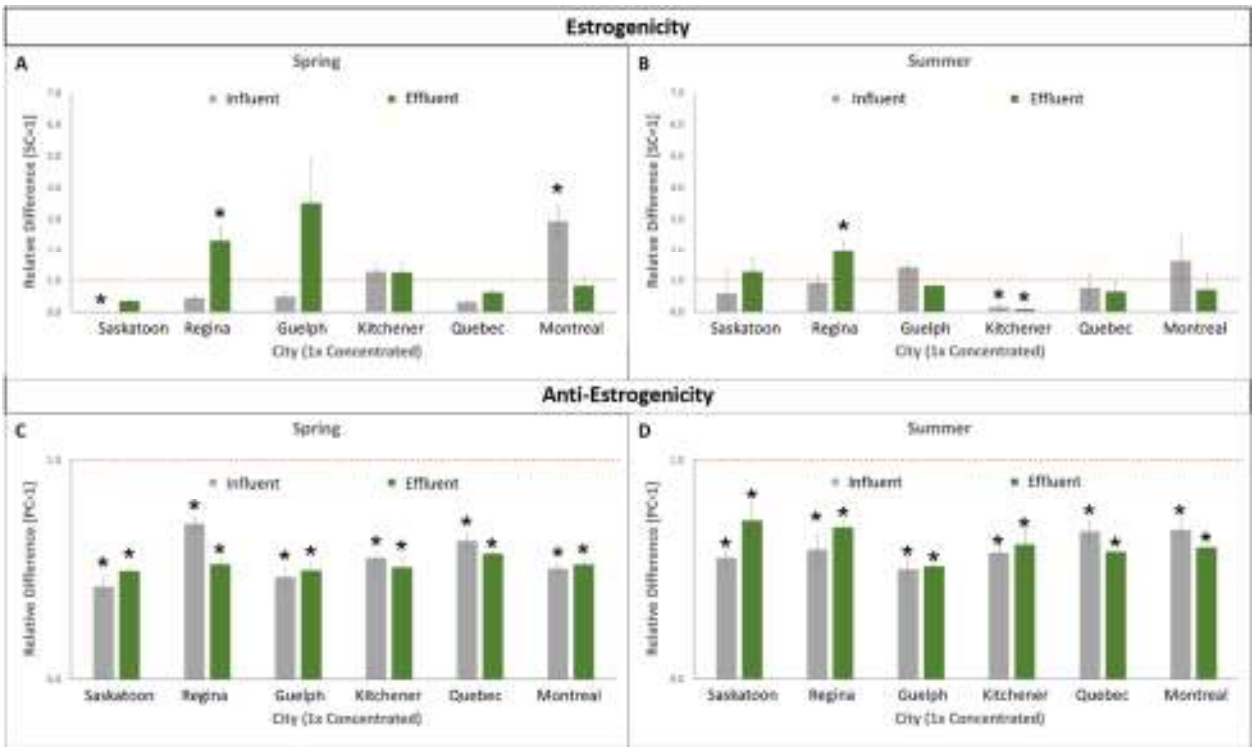

Figure 3.2 - Estrogenic (A and B) and anti-estrogenic (C and D) activity of extracts of influents and effluents (1x concentrated) collected from 6 WWTPs during spring (A and C) and summer (B and D) of 2014 determined using the MVLN *in vitro* assay. Estrogenicity is expressed as relative changes compared to the solvent control (SC). Anti-estrogenic activity is expressed as fold-change relative to the positive control (PC; 81.7 ng/L E2). Data are represented as the mean ± SEM (n=4, wells). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$). Data for Regina and Saskatoon are from chapter 2.

3.4.3 Androgenicity and anti-androgenicity

Most of all influent samples from the six WWTPs showed a significant increase in androgenicity when compared to the SCs, except for samples collected from Quebec City and Montreal during summer (Figures 3.3A and 3.3B). Samples of influents with the highest androgen activity were collected during spring from Regina, Kitchener and Quebec City with 10.2- (44.9 AEQ ng/L), 3.9- (13.4 AEQ ng/L) and 15.8-fold (86.3 AEQ ng/L) increase, respectively. During summer, influents from Saskatoon, Guelph, and Kitchener had the greatest androgenic activities with 11.9- (52.2 AEQ ng/L), 4.5- (18.3 AEQ ng/L) and 5.4-fold (23.1 AEQ ng/L) increases relative to SCs. In contrast, only a few effluent samples showed significant androgen activities. Specifically, influent collected during spring from Quebec City and Montreal caused 3.1- (6.9 AEQ ng/L) and 2.3-fold (12.7 AEQ ng/L) increases, and samples collected during summer from Montreal caused an 8.9-fold increase (2.2 AEQ ng/L) in androgenicity (Figures 3.3A and 3.3B).

Significant anti-androgenic potentials relative to the controls were only observed in influent samples collected in spring from Saskatoon and Guelph, with 0.1- and 0.4-fold changes relative to the positive control, respectively, and collected from Kitchener during summer with a 0.7-fold change. Most of the effluent samples sampled during spring showed (anti)-androgenicity, with 0.6, 0.8, 0.7 and 0.8-fold changes for Saskatoon, Regina, Guelph, and Kitchener, respectively. For samples collected during summer, only the effluent sample from Kitchener showed significant anti-androgenicity with a 0.7-fold change (Figures 3.3C and 3.3D).

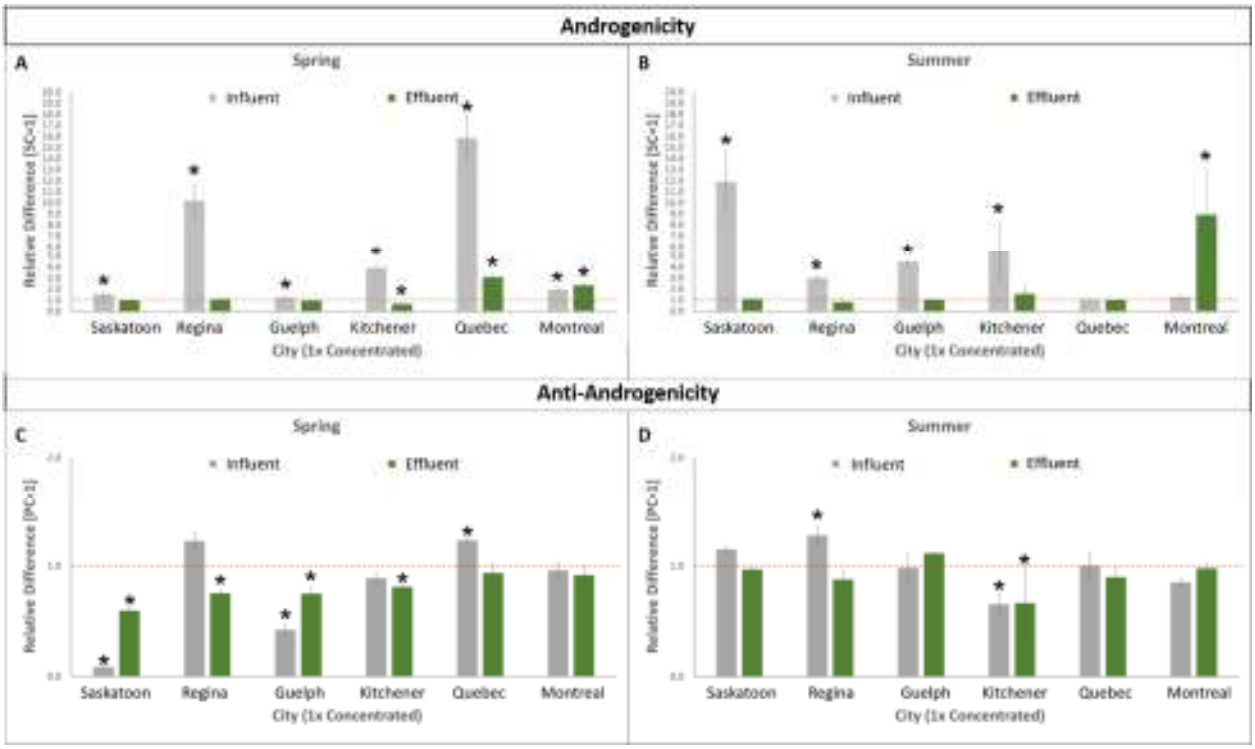

Figure 3.3 - Androgenic (A and B) and anti-androgenic (C and D) activity of extracts of influents and effluents (1x concentrated) collected from 6 WWTPs during spring (A and C) and summer (B and D) of 2014 determined using the MDA-kb2 *in vitro* assay. Androgenicity is expressed as relative changes compared to the solvent control (SC). Anti-androgenic activity is expressed as fold-change relative to the positive control (PC; 36.3 ng/L DHT). Data are represented as the mean ± SEM (n=4, wells). Dashed line represents baseline (controls). The asterisk (*) denotes a significant difference from the SC/PC ($p<0.05$). Data for Regina and Saskatoon are from chapter 2.

3.4.4 Steroidogenesis disruption

Some samples analyzed using the H295R assay affected the ability of extracts to modulate E2 synthesis; however, no consistent patterns were observed (Figure 3.4A and 3.4B). Only samples of influents collected from Saskatoon during spring and summer, and Montreal during summer showed a significant increase in E2 production with a 2.3-, 1.5- and 1.4-fold change, respectively. Furthermore, samples of effluents collected from Regina in spring and Kitchener in summer caused a 1.4- and 1.9-fold increase in E2 production, respectively. In contrast, some samples showed a significant decrease in hormone production when compared to the SCs, such as influent samples collected from Guelph, Kitchener and Quebec City during spring, ranging between 0.5- and 0.7-fold changes; and samples of effluent from Saskatoon, Guelph and Kitchener during spring, as well as, Regina and Quebec City during summer ranging between 0.4- and 0.6-fold changes.

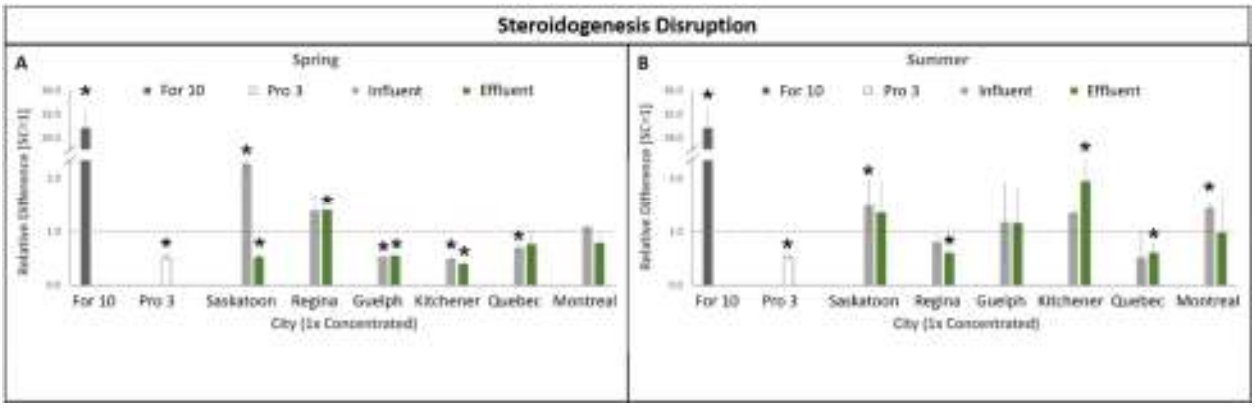

Figure 3.4 - Changes in 17β-estradiol production caused by extracts of influents and effluents (1x concentrated) from 6 WWTPs sampled during spring (A) and summer (B) of 2014 determined by H295R Steroidogenesis Assay. Data are relative changes compared to the solvent control (SC). Results are represented as the mean ± SEM (n=3, wells). The asterisk (*) denotes a significant difference from the SC ($p<0.05$). FOR forskolin [10 μM]. PRO prochloraz [3 μM]. *$p<0.05$. Data for Regina and Saskatoon are from chapter 2.

3.4.5 Chemical analysis

A total of 19 chemicals were detected during this study across all samples of influent and effluent from the six WWTPs, including prescription and non-prescription drugs, insecticides, herbicides, and other emergent contaminants related to wastewater. Most of the chemicals were detected at greater concentrations in influents compared to effluents. However, in some cases, the opposite was observed. In particular, some of the effluent samples collected from Quebec City and Montreal showed higher concentrations of chemicals than influents (Table S3.2). Among these contaminants, the greatest concentrations observed were for ibuprofen and naproxen (anti-inflammatory drugs), triclosan (antimicrobial disinfectant) and DEET (insecticide) at 41,618, 11,446, and 1,427 ng/L (Kitchener, influent), and 7,814 (Regina, influent), respectively (Table S3.2). While other chemicals were detected at lower concentrations, including carbamazepine (anticonvulsant) that ranged from 0.05 and 29.9 ng/L in the influents, and from 0.54 and 18.9 ng/L in effluent samples, as well as clofibrate (antihyperlipidemic) with concentrations from 27.9 and 63.4 ng/L, and 30.9 and 109.4 ng/L in influent and effluent samples, respectively. Gemfibrozil (antihyperlipidemic) was only detected in Guelph and Regina influent samples at 23.1 and 268.2 ng/L, respectively, and in Montreal and Quebec City effluent samples at 16.4 and 17.3 ng/L, respectively. Ibuprofen concentrations ranged from 202.7 to 41,618 ng/L in influents but were only detected in effluents of Quebec City and Montreal at concentrations between 194.1 and 1,637 ng/L. Naproxen was detected only in influents of Ontario and Quebec WWTPs (17.3-11,446 ng/L) and was only measured in effluents of Montreal and Quebec City at concentrations of 18.7 and 321.0 ng/L during spring and summer, respectively. Finally, DEET and triclosan were detected in influents and effluents of all treatment plants. DEET was detected at concentrations between 3.15 and 7,814 ng/L in the influents and between 6.98-3,168 ng/L in effluents, with significantly greater

concentrations during summer compared with spring. Triclosan was found at concentrations between 1.52 and 1,427 ng/L, and between 0.37 and 106.1 ng/L, in influent and effluents, respectively. Of the natural sex steroids, only progesterone (0.62-13.1 ng/L) and testosterone (93.4 ng/L; Guelph) were found in influent samples. In addition, progesterone and testosterone were also measured in effluent samples of Montreal during spring at concentrations of 7.47 ng/L and 1.58 ng/L, respectively. Concentrations of other reproductive hormones, such as E2, EE2, E1, and E3 were below the detection limit in both influent and effluent samples (Table S3.2). Overall, there were no clear patterns in the occurrence and/or concentrations of detected chemicals as a function of geographic region, urban demographics (population) or treatment level.

3.5 Discussion

Significant endocrine potentials were observed across influents and effluents from all six WWTPs studied using *in vitro* bioassay-based analyses. However, there were significant differences in the types and magnitudes of responses, which were likely due to differences in population demographics, types of wastewater treated, and level of treatment systems utilized in the respective WWTPs.

3.5.1 Estrogenicity and anti-estrogenicity

Except for effluent samples collected from Regina during both seasons (see chapter 2) and influents from Montreal during spring, none of the samples analyzed showed significant estrogenicity. In particular, estrogenic potentials detected in influents that ranged between <0.3 to 0.9 ng EEQ/L were much less than those detected by other studies conducted in China, the Czech Republic, Germany and Israel with EEQs ranging between 20 and 80 ng/L (Sui et al. 2010; Jálová

et al. 2013; Maletz et al. 2013; Shore and Shemesh 2016). Also, estrogenic potentials were in the lower range of those detected in influent from the Netherlands (1-120 ng/L), and Australia and New Zealand (<4-185 ng/L) (Leusch et al. 2006; Jálová et al. 2013). In contrast, EEQs detected in effluent samples, which ranged from 0.5 to 1.4 ng/L, were similar to those reported by some of the above authors, which reported values ranging from <1.0 and 5.1 ng/L (Leusch et al. 2006; Jálová et al. 2013). Furthermore, the relative changes compared to the controls observed for effluents in this study, which ranged from <0.5 - to 3.5-fold, were comparable to those observed by Maletz et al. (2013) who reported 1.5- to 2.9- and 2.0- to 3.8-fold increases in estrogenic activities determined using the LYES and ER CALUX® assays, respectively.

Effluent samples collected from the Regina WWTP showed higher estrogenicity than influents during spring and summer (see chapter 2) . Similarly, samples of effluent, but not influent, collected from the Guelph WWTP during spring showed a trend towards elevated estrogenicity. Studies such as Pessoa et al. (2014) and Kirk et al. (2002), reported that WWTPs utilizing lagoon-based treatments, such as Regina, had the lowest efficiencies for the removal of estrogenic hormones, ranging between 54 to 80% and 7 to 10%, respectively, which is in accordance with the lack of removal of compounds with estrogenic properties observed in this study. The trend towards increased estrogenicity for the Guelph effluent samples in spring was somewhat unexpected given the advanced system this plant operates under, and which includes a tertiary treatment step with conventional and extended activated sludge. However, during the time of sample collection, the Guelph WWTP was undergoing a significant upgrade to a tertiary treatment plant and had a series of construction issues (Hicks et al. 2017c), which may explain the reduced efficiency at removing estrogenicity (Figure 3.2A).

It is hypothesized that the weak or lack of estrogenic potentials in most WWTP samples was due to high concentrations of other and diverse contaminants present in complex mixtures with anti-estrogenic or general toxic potentials that were likely to have masked compounds binding agonistically to the estrogen receptor. Other studies made similar observations and suggested that chemicals with anti-estrogenic potencies, including pesticides such as linuron or atrazine that are commonly found in raw sewage and effluents, could be the cause of reduced or lack of estrogenic responses (Orton et al. 2009; Jálová et al. 2013). This is in accordance with the significant anti-estrogenic potentials that were reported for all samples in our study. For the Regina and Guelph WWTPs, it can be assumed that true anti-estrogenic potentials may have even been greater considering the remaining significant estrogenicity in the same samples. Similarly, Jálová et al. (2013) reported anti-estrogenic potency in extracts from eight sites studied in the Czech Republic, including influents and effluents, with greatest anti-estrogenicities occurring in effluent samples. Furthermore, in two parallel *in vivo* studies conducted by Steeves et al. (in preparation) and Hanson et al. (in preparation a,b), the authors observed no estrogenic effects in laboratory or wild fish exposed to Regina and Saskatoon MWWEs; however, significant and marked anti-estrogenic responses, such as significant decrease in egg production, delayed maturity and increase in oocyte atresia in the ovaries in females were observed. Together with observations by Hill et al. (2010) who observed a trend for increased anti-estrogenic activity in fish exposed to effluents, this supports the hypothesis that anti-estrogenic potentials may be of greater relevance in many MWWEs than agonistic effects mediated through the ER. In addition, a study conducted by Hicks et al. (2017a) that evaluated the endocrine disrupting effects of effluent samples from the Guelph and Kitchener WWTPs between 2007 and 2015 showed a significant reduction in the occurrence of intersex by 70-100%, which the authors attributed to the upgrades performed on the Kitchener

facility. This is in accordance with the lack of estrogenic responses observed during the in here-applied *in vitro* assays for effluents collected from these plants.

Furthermore, only chemicals with weak estrogenic properties, such as triclosan, were detected at very low concentrations that were much lesser (1.52-1,427 ng/L in influents and 0.37-106.1 ng/L in effluents) than those shown to cause effects (20,000 ng/L) (Ishibashi et al. 2004) while strong agonists including E1, E2, EE2, and E3 were not detected. This further supports the lack of or the weak estrogenic potentials observed during the *in vitro* tests. In contrast, a number of chemicals that were previously described to inhibit estrogenic responses were found both in influents and effluents, including atrazine (Orton et al. 2009), clofibrate (Fujimoto et al. 2012; Ezechiáš et al. 2016), ibuprofen (Ezechiáš et al. 2016) and naproxen (Ezechiáš et al. 2016) (Table S3.5). Atrazine was only detected in effluent samples from Montreal at concentrations 2- to 21-million-fold less than those previously reported to inhibit estrogenic responses (Orton et al. 2009). Therefore, this chemical was unlikely to be responsible for the anti-estrogenicity observed here. However, some other compounds, such as clofibrate (30.93-109.4 ng/L), ibuprofen (194.1-1,637 ng/L) and naproxen (18.73 and 321.0 ng/L; only detected in Montreal and Quebec City) occurred in some effluent samples at concentrations previously described to cause anti-estrogenicity; clofibrate at 10 ng/L, ibuprofen at 700 ng/L and naproxen at 50 ng/L, using the *in vitro* yeast-based YES assay (Ezechiáš et al. 2016). In conclusion, the absence or presence of weak inducers and significant presence of inhibitors detected by the chemical analysis is in accordance with what was observed by the *in vitro* assays.

3.5.2 Androgenicity and anti-androgenicity

Concentrations of androgens reported as AEQs observed in the present study (2.5-86.3 and <2.3-12.7 ng AEQ/L in influents and effluents, respectively) were in the lower range of or less than those reported previously by other authors (<200-9,330 ng and 0-736 ng AEQ/L in influents and effluents, respectively) (Kirk et al. 2002; Leusch et al. 2006; Sousa et al. 2010; Shore and Shemesh 2016). The relatively low androgenic potentials observed in this study may be explained by the relatively low population sizes served by most of the investigated WWTPs, which were below 250,000 inhabitants for most of the treatment plants, with exception of Quebec City and Montreal. Kirk et al. (2002) hypothesized that androgens excreted by humans are responsible for most of the androgenic activity in municipal effluent with a high domestic input, which was reflected by the observation that the highest androgenic potentials were detected in WWTPs in the UK that were serving large populations (>1 million inhabitants). This is in accordance with the findings of our study that reported the greatest androgenic potentials in influents and effluents of the Quebec City and Montreal WWTPs serving populations of >500,000.

For the majority of WWTPs, there was a significant decrease in androgen activity to baseline levels in effluents compared to influents. This is in agreement with earlier studies that suggested WWTPs are efficient at removing androgen-like compounds in general (Thomas et al. 2002; Svenson and Allard 2004; Hill et al. 2010;), as well as results from chapter 2. With the exception of Montreal, where greater AEQs were observed in effluents compared to influents, removal efficiencies of compounds with androgenic activities ranged from 64 and 81%, which is within the range of removal efficiencies of 7 to 99% previously reported by several other authors (Kirk et al. 2002, Leusch et al. 2005, Jálová et al. 2013). The highest removal efficiencies (90-97%) occurred in treatment facilities with secondary or greater treatment systems using activated sludge. However,

facilities with primary treatments, without activated sludge, had much lower removal efficiencies of approximately 7% (Kirk et al. 2002). A similar pattern was observed in the current study, where the lagoon-based Montreal WWTP showed no removal of androgenicity. In addition, despite the good-moderate removal efficiency (> 80%) observed by the Quebec City WWTP during spring, effluent samples from this WWTP, which does not include a nitrification treatment step, had significant remaining androgenic potencies during spring. The reduced removal efficiency of androgens from the Montreal WWTP may be also explained by the exposure to severe temperatures in the open lagoon system for extended periods of time, which may result in significantly decreased microbial activity during cold winters; and/or increased evaporation, and thus increasing concentration of contaminants, during hot summers (chapter 2; Pessoa et al. 2014). As Montreal treats combined storm- and waste-water flows, the greater androgenicity detected during summer compared to spring could potentially be due to higher dilution in the spring as a result of snowmelt events, which was similar to observations by Kirk et al (2002). Besides dilution and accumulation of contaminants due to the collection through stormwater and seasonal melting, in the case of heavy rain events, untreated sewage can bypass the WWTP and be discharged into the aquatic environment. In one such event in 2015, the Montreal WWTP discharged upwards of 4.9 billion liters of untreated effluent into the Saint Lawrence River (Ross, 2016). Interestingly, the Regina WWTP, which also used a lagoon as the secondary treatment step, did show good-moderate removal efficiencies of approximately 80%. This could potentially be due to the seasonal UV-sterilization (April-November) performed by the Regina WWTP, a step not used at the Montreal facility. Previous studies have demonstrated that UV-sterilization has the ability to increase photodegradation of some natural and synthetic hormones, including androgens (Liu et al. 2003; Liu and Liu 2004; Gryglik et al. 2010).

In conclusion, the significant androgenic potentials that remain in Montreal and Quebec City effluents may pose a threat to receiving environments. Previous studies have observed negative impacts on reproduction by fish exposed to androgens, such as 17-β-trenbolone (≥ 27 ng/L), levonorgestrel (≥0.8 ng/L), and levonorgestrel or Gestodene (100 ng/L) (Ankley et al. 2003; Zeilinger et al. 2009; Runnalls et al. 2013). While it is unclear how the potencies of the above androgens compare to the positive control used in this study, namely DHT, considering that DHT is among the most active androgens in mammals, the AEQs observed in effluents of the two Quebec WWTPs warrant further investigations into the potential environmental impacts in downstream organisms.

Interestingly, anti-androgenicity was only observed in a few influent samples. This lack of anti-androgenic responses was likely due to the very great androgenic potentials observed in most of these samples, and which is hypothesized to have masked the effects of contaminants with anti-androgenic activity. In contrast, significant anti-androgen potentials were detected in most of the effluent samples, particularly in spring, except for Quebec City and Montreal, indicating a limited capacity of the WWTPs to remove compounds with anti-androgenic properties. This is in agreement with Hill et al. (2010), who observed no androgenic activity in MWWE samples, but significant anti-androgenicity using the yeast-based YAS assay. The same pattern was not observed in samples collected during summer, where only Kitchener influent and effluent showed significant anti-androgenic activity. As also observed by Kirk et al. (2002) and results from chapter 2, samples collected during spring showed generally higher endocrine activities than samples collected during summer. Kirk et al. (2002) attributed this to greater microbial activity due to higher temperatures during summer months as well as to increased rainfall causing greater dilution of contaminants in the combined storm- and waste-sewer system, all of which vary seasonally.

The hypothesis that colder temperatures were responsible for the observed increase in anti-androgenic responses was unlikely the main reason for the observed greater potentials in this study as in a parallel study no elevated anti-androgenic potentials were observed for Regina and Saskatoon effluents during the much colder winter months when compared to summer (see chapter 2). Furthermore, none of the WWTPs investigated in this study, except for Montreal, treat combined storm- and wastewater flows, and thus, dilution was unlikely to have affected wastewater composition in a similar manner as described in Kirk et al. (2002). The lack of androgen inhibition of effluents observed for the Quebec City and Montreal treatment plants was likely due to the high remaining androgenicity, suggesting that effects of anti-androgens were likely masked by androgens present in the same samples. In contrast, Saskatoon and Guelph influent samples showed low androgenic activity and significant antagonistic activity during spring, suggesting that androgenic effects could have been masked by the presence of potent anti-androgens. This would be in agreement with observations by Weiss et al. (2009), who established that androgen receptor agonistic potency was masked by antagonist effects when testing sediment extracts.

Overall, the data suggest that anti-androgenic potentials represented a greater concern in this study than androgenic potentials, which is in agreement with two parallel *in vivo* studies investigating effects of Regina and Saskatoon MWWEs on the endocrine system of fish (Steeves et al. (in preparation); Hanson et al. (in preparation a,b). These studies revealed significant demasculinization of male fish, which would be in accordance with the strong anti-androgenic potentials reported by the current study.

Unlike estrogenic effects, androgenic and anti-androgenic effects have not been widely reported in wildlife to date; however, several studies have hypothesized that these types of disruptions may

be of environmental concern given that chemicals with (anti)-androgenic properties are commonly found in MWWEs (Johnson et al. 2007; Urbatzka et al. 2007; Jobling et al. 2009b; Hill et al. 2010). Although, chemicals with the ability to act as androgen agonists such progesterone, testosterone, and triclosan (Foran et al. 2000; Jenkins et al. 2003; Ishibashi et al. 2004; Dequattro et al. 2012; Zucchi et al. 2012; Sangster et al. 2016) were detected in the current study, none of these, with the exception of chemicals discussed below, were present at concentrations previously reported to cause androgenic effects, which is in agreement with the lack of androgenic potentials observed in effluents (Table S3.5). The one exception was effluent samples collected from the Montreal WWTP, and which contained 7.47 ng/L progesterone and 1.58 ng/L testosterone during spring, as well as 13.93-62.63 ng/L triclosan during both seasons, and which could potentially explain the androgenicity increase observed by the AR assay. In particular, progesterone concentrations were similar to previous studies showing androgenic effects at concentrations ranging from 2 to 10 ng/L (Jenkins et al. 2003; Dequattro et al. 2012; Zucchi et al. 2012; Sangster et al. 2016). However, concentrations of triclosan and testosterone were ~200- and ~400-fold lower than those previously reported to cause androgenicity *in vitro* (Foran et al. 2000; Ishibashi et al. 2004; Sangster et al. 2016), and thus, it is uncertain to what extent these chemicals could have contributed to the observed effects. Furthermore, this study identified a number of chemicals in effluents and influents that have previously been reported to act as anti-androgens including carbamazepine (Fraz et al. 2018), clofibrate (Cook et al. 1999; Fernandes et al. 2011; Fujimoto et al. 2012; Ezechiáš et al. 2016), gemfibrozil (Liu et al. 1996; Mimeault et al. 2005), ibuprofen (Han et al. 2010; Ezechiáš et al. 2016), naproxen (Ezechiáš et al. 2016) and DEET (Zenobio et al. 2014). However, of these compounds, only DEET occurred at concentrations that have previously been

observed to act as anti-androgens (600 ng/L; Table S3.5; Zenobio et al. 2014), while all other compounds were measured at concentrations below effective levels.

3.5.3 Steroidogenesis disruption

Overall, only a few samples influents or effluents from the six WWTPs investigated by this study showed a significant increase or decrease in E2 production. The effects of exposure to forskolin (positive control) and prochloraz (negative control) on the production of E2 (30-fold increase and a 0.5-fold decrease, respectively) met the performance criteria of ≥7.5-fold increase and ≥0.5-fold decrease, respectively, set forth in the OECD H295R guideline (OECD Test No. 456, 2011), demonstrating that the assay performed as expected. The magnitudes of effects were comparable to those reported by Maletz et al. (2013) after exposure of H295R cells to MWWEs in Germany, and which rarely exceeded 2-fold. Significant increases in E2 concentration in samples of influents from Saskatoon during spring and samples of effluents from Kitchener during summer indicated that these samples contained compounds with the ability to stimulate E2 production. In contrast, the majority of other samples, particularly effluents, that revealed altered sex steroid synthesis in H295R cells, inhibited hormone production. Thus, there was no clear pattern with regards to the presence of compounds that disrupt steroidogenesis either as a function of treatment level, population size or climate. It is hypothesized that this variation in responses observed in the current study was likely associated with the complex nature of the investigated wastewaters, where seasonal factors such as temperature, evaporation, photoperiod and daily flow changes were likely to have affected the different compounds in the complex mixtures in unique ways. Previous studies also observed unpredictable responses when chemicals individually known as steroidogenesis inducers or inhibitors were mixed, emphasizing that these chemicals do not always respond as

predicted when present in a complex mixture and that one chemical alone may not be responsible for the responses observed (Heindel et al. 1995; Olmstead and LeBlanc 2005; Hadrup et al. 2013b). Moreover, Gracia et al. (2008) reported high variability in the response of H295R cells to the exposure with MWWEs from different WWTPs in Hong Kong, SAR, China ranging from no effects in E2 production for most effluent samples to significant inhibition and induction of E2 synthesis by one influent and effluent, respectively. The authors suggested the increased E2 production in response to the effluent from the one WWTP (San Wai) was likely due to the outdated facility not being able to effectively remove EDCs with steroidogenesis disrupting properties. However, a similar pattern indicating that less advanced treatments systems, such as lagoon-based systems, have lesser efficiency to remove contaminants with steroidogenesis disrupting properties was not observed in this study. Consequently, it is uncertain what step of the wastewater treatment process contributed to the elimination of compounds with estrogen production promoting properties.

Overall, few studies that investigated the potential impacts of MWWEs on E2 production are available to date, and the results of this study reflect the overall variation within and among studies in terms of the direction of responses and their magnitude (Gracia et al. 2008; Hadrup et al. 2013b; Maletz et al. 2013). Thus, it remains challenging to pinpoint the specific factors or chemicals that drive the steroidogenesis disrupting potentials of MWWEs. This is also in accordance with the results obtained with the receptor-mediated assays, which demonstrated that MWWEs contain complex mixtures of EDCs with continuous change in flow, chemical concentration, composition, and removal efficiency, resulting in varying effects on endocrine endpoints.

Results obtained by chemical analysis identified few chemicals that have been previously reported to affect E2 production. One such compound is ibuprofen, which has been shown to increase E2

production at concentrations ranging between 20,000 and 200,000 ng/L using the H295R assay (Han et al. 2010) (Table S3.5). In general, concentrations of this chemical in influents (<MDL-8,813ng/L) and effluents (<MDL-1,637 ng/L) were less than effect concentrations in H295R cells. The exception was influent collected in summer from the Kitchener WWTP, with concentrations of 41,618 ng/L. However, this sample did not affect E2 production. Therefore, the observed increase in E2 production was unlikely to have been caused by the presence of ibuprofen, and it remains unclear what the specific chemicals or mixtures responsive for the measured effects were.

Despite the variation in responses in E2 production, clear signs of disruption of steroidogenesis were observed, indicating that the steroidogenesis disruption assay represents an important component for EDC screening. Specifically, the H295R Steroidogenesis Assay has been shown to be a reliable and relatively cost-effective screening method, providing important information regarding alteration of the endogenous synthesis pathways of sex steroids (Villeneuve et al. 2007; Ji et al. 2010; Hecker et al. 2011).

3.6 Conclusion

This study illustrated that WWTPs across the three provinces of Canada studied represent significant sources of compounds with endocrine active properties to surface waters, albeit the extent and type of the observed potentials varied greatly among the WWTPs as a function of treatment level, seasonality, and population size. Although most WWTPs investigated were highly efficient in removing compounds with androgenic properties, even most advanced WWTP systems were limited in their efficiency to remove compounds with anti-androgenic and anti-estrogenic potentials, regardless of the treatment level. Surprisingly, with exception of Regina, neither estrogenic potentials nor significant concentrations of compounds with estrogenic properties were

observed using a combination of bioassays-directed and chemical analyses, suggesting that estrogenicity does not seem to be a major concern in WWTPs in Central and Eastern Canada. Furthermore, the lagoon-based Montreal WWTP and the secondary treatment facility from Quebec City were the only ones that had significant remaining androgenic potentials in effluents. Concerns are particularly warranted in cases where the effluent flow is proportionally greater than that of the receiving water, as is the case of Wascana Creek, SK, and for which significant effects in resident fish population were observed, or where the population is greater than WWTPs' capacity of treatment, as in the case of Montreal. In addition to treatment level and population demographics of the WWTPs investigated, seasonality also seemed to have affected the removal efficiency of certain compounds with endocrine potentials, in particular during spring. The highly complex mixtures present in influents and effluents of WWTPs, including agonists, antagonists, and cytotoxic components may explain the high variability in response patterns observed in the applied bioassays. To conclude, treatment level, seasonality, and population size seemed to play an important role regarding EDC removal, where treatment level and population size appeared to be the main factors driving the presence of chemicals with endocrine properties. Summary of results can be found in Table 3.1. Given the complex nature of the effluents analyzed, future studies should employ experiments that expand the endpoints utilized. Moreover, the significant remaining anti-estrogenic and anti-androgenic potentials regardless of treatment level warrant further examination of the effectiveness of current WWTP technologies to remove endocrine active compounds and assessment of the associated risks to receiving aquatic environments.

Table 3.1 – Summary of *in vitro* results describing the endpoints and outcomes of potential risks of MWWEs from all six WWTPs, detected by MVLN (anti-)estrogenicity, MDA (anti-)androgenicity, and H295R (steroidogenesis disruption). YES, effluent from at least two WWTPs showed significant activity during both seasons. SOME, effluent from a minimum of one of the WWTPs showed significant activity at least during one season.

Are MWWEs a significante source of EDCs?		
Endpoint	**Outcome**	**MWWEs**
ER	SOME	Regina
Anti-ER	YES	All six WWTPs
AR	SOME	Quebec City and Montreal
Anti-AR	SOME	Monstly from SK and ON WWTPs during spring
Steroidogenesis disputors	SOME	Most of the WWTPs affected production of E2, with effluents causing decrease of E2 overall. However, no consistent pattern was observed
Does treament level affect EDC removal?	SOME	Primary treament plants showed greater endocrine activity overal (Regina and Montreal)
Does population size affect EDC removal?	SOME	Treament plants with population > 500,000 people showed greater endocrine activity overal (Quebec and Montreal)
Does seasonality affect EDC removal?	SOME	Spring showed greater endocrine activity overal
Can *in vitro* assays be used as a cost-effective tool for prioritizing potential endocrine disrupting impacts of MWWEs	YES	Results from parallel *in vivo* and chemical analysis studies, were in agreement with *in vitro* findings

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CHAPTER 4: GENERAL DISCUSSION

4.1 Introduction

The occurrence of EDCs in aquatic environments has become a subject of increasing concern over the past decades due to their potential to adversely affect human and wildlife (Hughes et al. 2013; Gavrilescu et al. 2015). Large quantities of EDCs are released into surface waters through municipal wastewaters as a result of bodily excretion of natural hormones, unmetabolized pharmaceuticals, indiscriminate disposal of unwanted/expired pharmaceuticals and personal care products (PPCPs) as well as many other household products (Jobling et al. 1995; Daughton and Ternes 1999; Lahnsteiner et al. 2005). This is because traditional wastewater treatment technologies are designed to mainly remove organic nutrients, nitrate, and phosphorus; however, removal or breakdown of EDCs during the wastewater treatment process is either incomplete or inexistent, and thus, MWWEs are considered a major source of these compounds in the aquatic environment (Vieno et al. 2007; Lapworth et al. 2012; Luo et al. 2014b; Hai et al. 2016). Due to the multitude of mechanisms of actions and effects of EDCs, which can be attributed to various classes of chemicals this group of contaminants consists of, identification of these chemicals in complex mixtures such as MWWEs that are responsible for reproductive alterations in whole organisms can be challenging and requires the development of specific and integrative procedures for environmental risk assessments (Grund et al. 2011). Several studies have demonstrated that the combination of receptor and non-receptor-mediated *in vitro* assays and targeted chemical analysis represents a promising approach to address this challenge (Giesy et al. 2002; Grund et al. 2011; Maletz et al. 2013). Therefore, the main objective of the research conducted in this thesis was to identify and quantify EDCs with, (anti-)estrogenic and (anti-)androgenic and steroidogenesis

disruption potencies in MWWEs using a battery of *in vitro* assays and correlating the results to targeted chemical analysis, as well as to two parallel *in vivo* studies.

The first study focused on evaluating MWWEs from two WWTPs in Saskatchewan, Canada, regarding their efficiency to remove compounds with endocrine disrupting properties, as well as to characterize the potential effects that extreme climatic conditions typical for the northern prairies may have on this process. From this study, we observed that in general both the Regina and the Saskatoon WWTPs ameliorated androgen potencies; however, the less advanced treatment plant of the city of Regina was less efficient at removing compounds that act as estrogens. Furthermore, both treatment plants were unable to completely remove ER and AR antagonists, which were still present at significant levels after treatment, thus remaining a major concern to receiving aquatic environments. Moreover, seasonality appeared to affect EDC removal, with spring showing greater endocrine activities in general when receptor and non-receptor mediated bioassays where compared. The second study was designed to investigate EDCs from six WWTPs across Canada with different treatment levels, population size, and climate. This study confirmed that in general androgenic activities significantly decreased after treatment by most WWTPs; however, less advanced treatment systems such as Montreal and Regina were less efficient at removing compounds with androgenic and estrogenic properties, respectively. This demonstrated that lagoon-based treatments plants, which are still common in many parts of Canada, are not as efficient at removing EDCs from wastewater compared to advanced secondary or tertiary systems. In addition, all six facilities showed significant remaining ER and AR antagonistic potentials regardless of treatment system, emphasizing that traditional WWTPs are limited regarding their ability to remove EDCs, particularly in regard to inhibitory compounds (Clara et al. 2005; Johnson et al. 2007; Benotti et al. 2009; Hecker and Hollert 2009). In addition, population size appeared to

have an impact on EDC removal efficiency, where WWTPs that served populations >500,000 habitants were less efficient at removing EDCs, in particular of compounds with androgenic properties. Interestingly, annual variation in climatic conditions across the different provinces studied did not appear to significantly affect EDC removal efficiencies as originally hypothesized with colder months affecting treatment efficiencies due to reduced biological treatment efficiency. However, similarly to what was observed in the Saskatchewan study, spring showed the least removal of compounds with endocrine properties when comparing results from all three cell lines.

4.2 Treatment level efficiencies at removing agonist and antagonist EDCs

As discussed within the previous chapters of this thesis, in order to allow more objective ecological risk assessments of MWWEs, there is a need to identify the main factor or factors that contribute to the release of EDCs through effluents, including treatment level, seasonal changes in climate and population size. Several studies have evaluated the efficiency of different treatment techniques in removing endocrine potencies, particularly regarding estrogen removal (Villeneuve et al. 1997; Ternes et al. 1999; Wilson et al. 2002, 2004). However, treatment factors that drive elimination of compounds with other endocrine properties such as androgenic, anti-estrogenic and -androgenic activities, as well as steroidogenesis disruption have not been characterized very thoroughly to date. The results from this study confirmed that more advanced WWTPs appear to efficiently remove androgen agonists from raw sewage and that less advanced treatment facilities were unable to completely remove AR and ER agonists, as in the case of Montreal and Regina WWTPs, respectively. More importantly, most of the WWTPs were not efficient at removing compounds that antagonistically bind to the AR and ER, regardless of treatment level. This was potentially due to the strong presence of a wide variety of antagonists at significant concentrations, masking

agonists compounds. In other words, both agonistic and antagonistic of the ER and AR were present in influent samples, competing for the same receptors, and that treatment may have mainly removed agonistic acting compounds. Thus, inhibitory action at the AR and ER appear to be the greatest concerns associated with the MWWEs across Canada. This finding was also reflected by parallel studies investigating the effects of the same effluents or downstream environments on fish endocrine system (Steeves et al., in preparation; Hanson et al., in preparation a,b). The lack of significant estrogenicity in most of the influents and some estrogenic activity in the effluents could also be due to deconjugation and conjugation of natural and synthetic hormones. Particularly, the estrogenic activity in aquatic environments has been primarily correlated with the presence of free steroid estrogens due to incomplete removal by WWTPs. Most steroids are excreted in their conjugated, and thus inactive, form (Desbrow et al. 1998; Gomes et al. 2003). However, some of these steroids, particularly E1, E2, EE2, and E3, have been reported to be deconjugated by the metabolic actions of bacteria found in wastewater systems and lagoons, and as a result, these compounds can become reactivated (Baronti et al. 2000; Johnson et al. 2000; Gomes et al. 2009; Kumar et al. 2012). Thus, this may explain the lack of agonistic activities in influents, and the stronger agonistic potentials observed in effluents, particularly in the case of estrogenicity.

4.3 Influence of seasonality and population size in the occurrence of EDCs

Overall, limited information is available regarding seasonal variability of removal efficiency of EDCs from wastewater, particularly for compounds with androgen receptor and steroidogenesis disrupting properties. This study is one of a few that investigated the seasonal variation in removal efficiencies of hormonally active substances from wastewater as a function of climatic conditions. Although a clear seasonality pattern was not observed for each *in vitro* assay individually, when

all three receptor and non-receptor mediated assays were compared, exposure to effluents collected during spring consistently resulted in the greatest effects, thus indicating least removal efficiency of EDCs. Several reasons have been hypothesized to explain these results. One possible reason for the elevated endocrine potencies measured during this season was the potential for reduced biological activity during the cold winter months prior to spring, especially for the lagoon-based Regina WWTP, likely decreasing biodegradation of compounds by microorganisms and/or light. However, the same pattern was not observed during early and late winter, which were also cold sampling periods. Surprisingly, in some cases, and in particular for ER-mediated responses, there was even an increase in endocrine activity during summer. The latter could potentially have occurred due to high evaporation events resulting in an increase in concentration, especially affecting open lagoon-based systems, and which was also observed by other authors (Pessoa et al. 2014). Therefore, the hypothesis that colder months would have the potential to adversely affect the removal of EDCs was not always supported by the results, and future research should include multiple seasons from all WWTPs analyzed to confirm the patterns observed in the present study. Furthermore, one of the difficulties of accurately quantifying removal efficiencies of EDCs by WWTPs is retention time, where depending on the length of time taken by the WWTP to process raw sewage, samples of effluent analyzed are often not directly representative of the initial influent. Some studies have been adopting the use of chemical markers, such as acetaminophen, carbamazepine, caffeine, DEET, and salicylic acid to trace the incoming raw sewage through the treatment process (Nakada et al. 2008; Tran et al. 2013, 2014). The quantification of chemical markers is performed by using isotopically labeled internal standards in the raw sewage, which allows for the detection of these chemicals during treatment processes, including in the final effluent (Tran et al. 2013). As these chemical markers are very stable and are not degraded during

treatment, they can be used to follow raw sewage through the whole process until it becomes treated effluent, reducing the possibility of misrepresentation of removal efficiencies. Although the results found here were similar among collection days and were in agreement with previous studies, future studies should include the use of chemical markers in order to avoid uncertainties related to such issue. In addition, another factor that appeared to be correlated with certain endocrine activities in MWWEs was population density. The results from this study suggested that WWTPs that served greater populations (>500,000 habitants) also presented significantly greater endocrine activities, in particular, androgenicity. However, as only two WWTPs served greater populations then 500,000 in this study, including Montreal with a primary lagoon system and Quebec City with secondary treatment step, future studies should include more treatment facilities with advanced technologies, along with high population density in order to confirm the results observed here.

4.4 Comparison of *in vitro* responses with *in vivo* studies

In addition to their ability to characterize exposure to certain biologically active chemicals in complex mixtures, *in vitro* approaches are increasingly seen as a compelling and relevant alternative to the use of live animals for chemical screening and prioritization as they address several ethical and economic concerns associated with animal testing (Jeffcoate 1996; Meager 2006; Hecker et al. 2011). However, there are a number of concerns regarding the direct predictivity of *in vitro* tests for *in vivo* effects, as *in vitro* tests often do not capture toxicokinetic processes such as metabolic elimination or activation of compounds, among others. Furthermore, *in vitro* tests use isolated cells from individual tissues that do not include interactions among tissues such as endocrine feedback loops that may occur under *in vivo* conditions. However, when *in vitro*

results obtained in chapter 2 were compared to those of two parallel *in vivo* studies that investigated the effects of Regina and Saskatoon effluents to the endocrine physiology of a native fish species (fathead minnow, *Pimephales promelas*), there was evidence of a direct correlation between responses observed with *in vitro* and *in vivo* systems. The low or lack of estrogenic or androgenic potentials of effluents reported by the *in vitro* assays was in accordance with the fish studies that revealed no significant effects that could be linked to exposure with estrogens or androgens such as induction of vitellogenin and occurrence of testicular oocytes in males or expression of secondary male sex characteristics in females, respectively (Steeves et al., in preparation; Hanson et al., in preparation a,b). In contrast, fathead minnows exposed to both Regina and Saskatoon MWWEs showed general inhibition of reproductive functions such as delayed maturation, degeneration of gonadal tissues, reduction in the expression of secondary sex characteristics, and in the case of the Regina WWTP, significant reduction of fecundity, which correlated with the predominant antagonistic potentials detected by the ER and AR receptor assays. Furthermore, the fact that greater *in vitro* endocrine activities were observed during spring season may constitute a significant additional ecological concern, as this time coincides with the spawning season of many fishes. Overall, the results obtained during this study suggest that responses observed with the different *in vitro* assays applied here not only can be predictive of *in vivo* effects but also inform and prioritize environments at greater endocrine risks.

4.5 Comparison of *in vitro* responses with the chemical analysis studies

Previous studies have demonstrated that even the most comprehensive chemical analyses can only explain a limited fraction of the biologically effective endocrine disrupting potential of complex environmental mixtures such as MWWEs (Heindel et al. 1995; Olmstead and LeBlanc 2005).

Therefore, the combination of *in vitro* assays and targeted chemical analysis has been proposed as a preferred and more efficient approach to pinpoint potential causative agents and characterize the overall endocrine disrupting potential of complex mixtures (Giesy et al. 2002; Grund et al. 2011; Maletz et al. 2013). This study compared results from *in vitro* assays with chemical analysis, and in general, the results obtained by the targeted chemical analyses aligned with the endocrine activities observed in the *in vitro* assays. Specifically, the lack of agonistic potentials observed in the ER transactivation assay was in accordance with the failure of the chemical analytical analysis to detect female reproductive hormones including E2, EE2, E1 and E3 or other chemicals with the ability to act as estrogens, and which have been previously identified as the main causative agents for estrogenic activities of MWWEs. However, a number of chemicals that were previously described to have anti-estrogenic or -androgenic properties were found at biologically active concentrations. These compounds included clofibrate, ibuprofen, and naproxen, as well as chemicals with anti-androgenic properties, including DEET, which may potentially be responsible for some of the inhibitory effects observed in the *in vitro* bioassays. Although targeted chemical analysis allowed us to analyze multiple compounds in complex extracts, this analytical method has some limitations, as the detection of some compounds in complex mixtures can be difficult without extensive sample pre-treatment. Furthermore, the absence of other contaminants commonly found in wastewater, such as acetaminophen and diclofenac (pharmaceuticals) (Carballa et al. 2004; Focazio et al. 2008), could be due to extract samples containing compounds that were not included in our internal standards (Table S2.6), as they were not analyzed. To better understand what specific compounds may have been responsible for the biological activities observed in this study, and isolate active agents, future studies should include advanced analytical steps, such as sample clean-up; including the use of a more target-specific extraction cartridge and fractionation, where

each fraction could be more easily analyzed and targeted compounds could be more accurately quantified. However, for the scope of this project, further sample treatment was not performed. Nonetheless, this study obtained promising data to support that the combination of *in vitro* assays and chemical analysis are a useful tool for supplementing chemical analytical characterization for the evaluation of environmental samples for EDCs (Ankley et al. 1998; Matthiessen and Johnson 2007).

4.6 Limitations and future research

There are several limitations within the studies conducted in this thesis that could be expanded on to provide further insights into the outcomes observed. Although results comparing both treatment level and seasonality showed some variability, this study demonstrated that in general, less advanced treatment facilities showed reduced removal efficiency of chemicals with endocrine active properties. While seasonality appeared to have affected removal of endocrine activities to some extent, with spring showing the greatest activities within all three bioassays and across treatment facilities, there are some remaining uncertainties regarding the hypothesis that colder temperatures may impact removal efficiencies by WWTPs as samples collected during early and late winter in Saskatchewan did not show comparable patterns. When it comes to removal efficiency, one of the limitations of evaluating endocrine removal is to track the effluent resulting from the processed influent as discussed above. Therefore, future studies should include some chemical markers, such as carbamazepine and caffeine to determine whether the effluent being discharged from the treatment plants is representative of the influent analyzed, particularly for WWTPs with long retention times (few to several months) where the effluent may not be representative of the same season as the influent.

Results also revealed greater androgenic activities in effluents of WWTPs serving greater populations (in exceedance of 500,000 inhabitants), and which has also been observed by Kirk et al. (2002). However, the sample size to derive these trends was small (only two WWTPs with population sizes >500,000). Therefore, future research should include more treatment plants with similar climate and level of treatment to better characterize the direct effect population size may have on treatment efficiency.

Results from the *in vitro* and the two parallel *in vivo* studies demonstrated that neither estrogen nor androgen receptor agonists appeared to be a significant concern in the majority of MWWEs investigated. Instead, ER and AR antagonists were the primary drivers of endocrine potentials in MWWEs, and which was confirmed by two parallel fish endocrine physiology studies (Steeves et al., in preparation; Hanson et al., in preparation a,b). Therefore, the combination of *in vivo* and *in vitro* studies provided comprehensive insights into the factors affecting whole organisms living in downstream environments of WWTPs, particularly in small water bodies such as Wascana Creek that provide little dilution of effluents. However, given the general inhibitory responses observed in fish exposed to Regina and Saskatoon MMWEs or collected from Wascana Creek, there is the possibility that chemicals other than those with anti-androgenic or -estrogenic potentials could have contributed to the observed effects. Therefore, to gain a more comprehensive understanding of the different drivers of the toxicities observed, future studies should employ experiments that expand the endpoints utilized by analyzing effects on additional steroidogenic processes including androgen or corticosteroid production, as well as general toxicity markers such as oxidative stress, genotoxicity, neurotoxicity and metabolic toxicity, among others. The results observed throughout this research were supported by *in vivo* findings obtained during two parallel studies (Steeves et al., in preparation; Hanson et al., in preparation a,b). However, *in vitro* assays have some

limitations with regard to predicting effect in whole organisms, including lack of interaction among tissues, such as endocrine feedback loops, as well as lack of toxicokinetic processes responses, such as metabolic elimination or activation of compounds that may be observed under *in vivo* conditions. Therefore, future studies should include development and optimization of *in vitro* assays to include these outstanding aspects (e.g. toxicokinetic modeling or metabolic steps). Furthermore, due to the limitations described here, to date there is still need to include *in vivo* components to correlate with *in vitro* studies to assess the true risks associated with the exposure to contaminants contained in effluents. Hence, future research should continue to use *in vivo* tests, and perhaps include the use of organisms, such as mammals, birds and fish that may show different sensitivity to the endocrine disruptors found within this study to confirm the results observed here. Additionally, the significant remaining ER and AR antagonistic potentials found across all WWTPs investigated, regardless of their treatment level, require further investigations to determine how effective even more advanced quaternary WWTP treatment technologies (e.g. membrane reactors, ozonation) are at removing chemicals with such properties to better understand associated risks to receiving water bodies.

Targeted chemical analysis results aligned with what was observed in the *in vitro* studies revealing the presence of compounds that can potentially act as ER and AR antagonists, while low or no presence of hormones or chemicals that can act as agonists at the ER and AR occurred. As the significant anti-estrogenic and anti-androgenic activities observed in most of the effluent samples were unlikely caused by only one or two of the individual chemicals detected by the targeted chemical analysis, there is also need to characterize the potential combinatory effects of the plethora of compounds present in the investigated MWWEs. Therefore, given the complex nature of the effluents analyzed and to better understand the specific components of MWWEs driving

toxicity, future studies should also include sample fractionation, such as fractionating sample extracts by e.g. polarity followed by *in vitro* bioassays, in order to be able to better pinpoint the types of chemicals and combinations of chemicals potentially responsible for the observed effects.

Finally, as this study showed variable effects with many different layers of information, including different endocrine endpoints, varying treatment levels across WWTPs, seasonality, and different population sizes, the statistical approach utilized by this study that only applied a one-way ANOVA with pairwise comparisons (SCs and treatment groups) has its limitations. Thus, future studies should submit the data generated by my study to more sophisticated statistical analysis, such as mixed regression models and/or multiple-way ANOVAs considering temperature, population size, etc. as co-factors. Such an analysis may help identifying key (confounding) factors that affect the efficiency of WWTPs to remove EDCs from raw sewage, and thus, affect the release of endocrine active compounds into Canadian surface waters.

4.7 Conclusion

This research successfully demonstrated that MWWEs are significant sources of EDCs to surface waters across Canada. Moreover, estrogenicity and androgenicity did not appear to be a main concern for the majority of effluents investigated; however, highly significant anti-estrogenic and anti-androgenic potentials remained in treated effluents of most of WWTPs regardless of treatments system, which was reflected by effects observed in fish living in downstream environments. In contrast to many other studies that focused on estrogenicity as the main endocrine property of concern (Kümmerer 2001; Carballa et al. 2004; Clara et al. 2005; Díaz-Cruz et al. 2009; Zorita et al. 2009), this study demonstrated that other inhibitory effects appeared to be of greater concern across Canada, which could be due to the interaction of the diverse chemicals in

mixtures potentially resulting in synergistic or antagonistic effects (Matthiessen and Johnson 2007), and thus, should be given more attention. Considering these inhibitory endocrine potentials remain in wastewater after treatment, there is a significant concern regarding the potential impacts of effluents from WWTPs due to incomplete removal of endocrine active compounds on downstream receiving environments. This is particularly true in cases of primary and outdated lagoon-based treatment systems, as well as in situations in which effluent flow is proportionally greater than the flow of the receiving water (e.g. Regina), which is the case of many urban municipalities in semi-arid regions such as southern Saskatchewan (Waiser et al. 2011a), or in situations in which the population is greater than WWTPs' capacity of treatment, as in the case of Montreal.

Although seasonality seemed to play an important role regarding endocrine activity, treatment level and population size appeared to be the more significant aspects influencing EDC removal. However, given the complex nature of effluents, sources, and climate, future research warrants a more integrative approach including a greater diversity of endpoints, including more complete characterization of effects along the steroidogenic pathway (e.g. androgen or corticosteroid production), and other general toxicity endpoints such as oxidative stress, genotoxicity, etc. In addition, future studies should be conducted at the Regina facility, which has recently undergone a significant upgrade, to assess whether endocrine removal has improved, and potentially confirm whether more advanced treatment level can efficiently remove endocrine activity. Moreover, it is recommended to apply advanced effect-directed analysis steps, such as sample fractionation to better-enabling pinpoint specific chemicals responsible for the observed effects.

Overall, this study successfully demonstrated that *in vitro* assays are a cost-effective tool to predict EDCs in complex mixtures, and results from *in vitro*, *in vivo* and targeted chemicals analysis were

found to be generally in agreement. To conclude, all three *in vitro* assays could be used as a first screening tool for the identification of EDCs in an environment in question, especially when attempting to analyze complex mixtures. This would not only help with the identification and quantification of EDCs in MWWEs but also allow regulators to prioritize specific treatment plants when reviewing future upgrades, with the potential of less investment in time, cost and use of live animals.

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APPENDIX: SUPPLEMENTAL FIGURES AND TABLES

Supplemental figures and tables are included in this chapter. The figure or table is presented x.y, where x indicates chapter number; y indicates figure or table number.

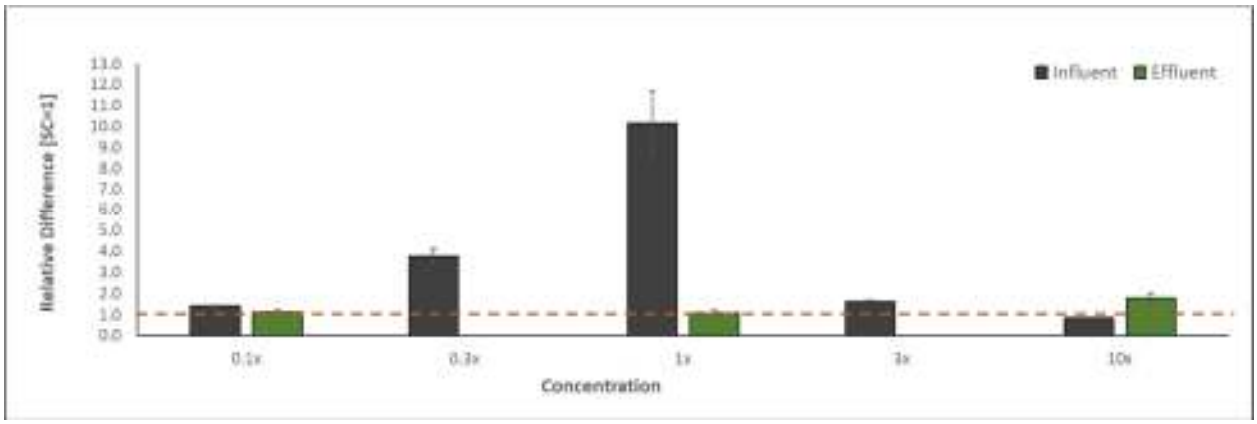

Figure A2.1 - Example of dose-response expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents at 0.1x, 0.3x, 1.0, 3.0x and 10x concentrations from Regina during spring determined by MDA-kb2 *in vitro* Assay. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

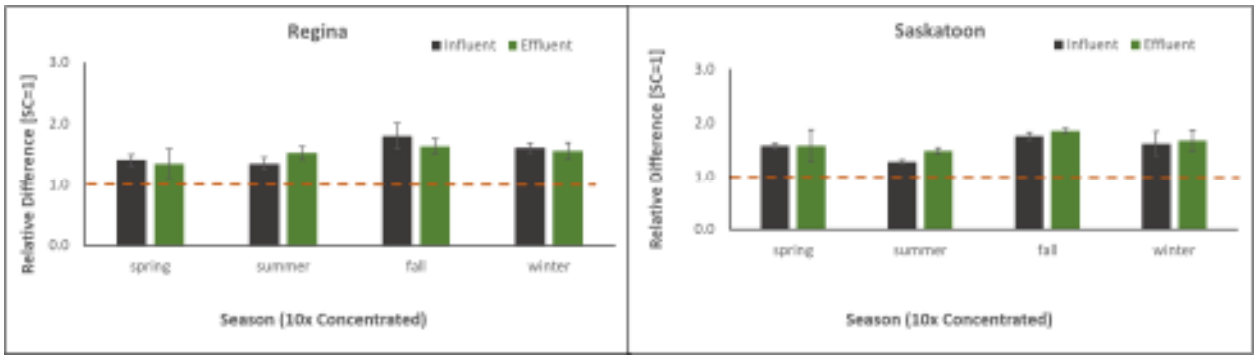

Figure A2.2 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by MVLN *in vitro* Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

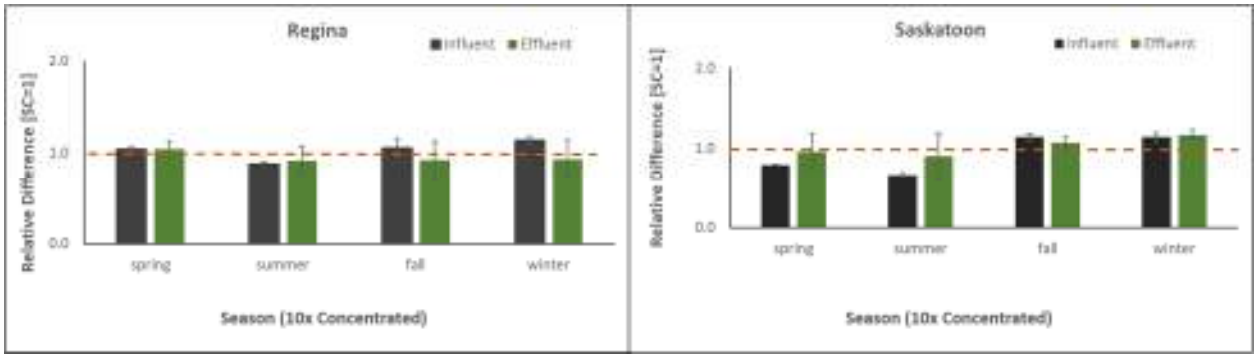

Figure A2.3 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by MDA-kb2 *in vitro* Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

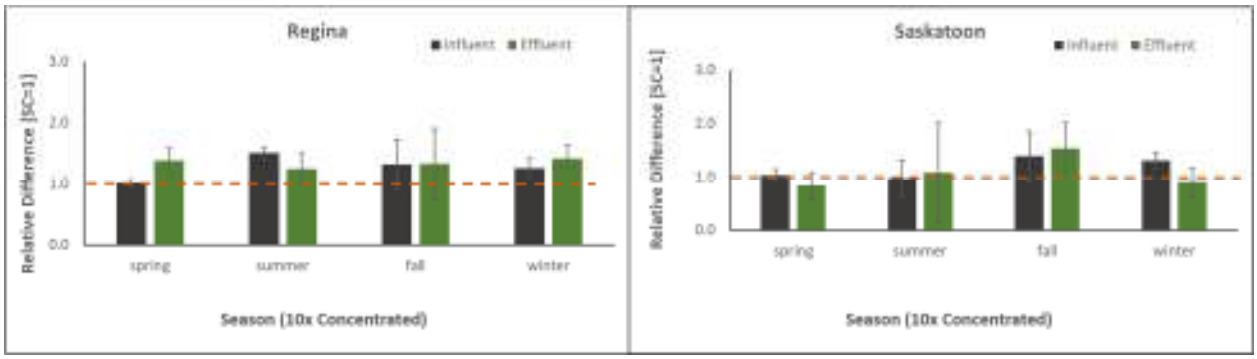

Figure A2.4 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from Regina and Saskatoon determined by H295R Steroidogenesis Assay during four seasons. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

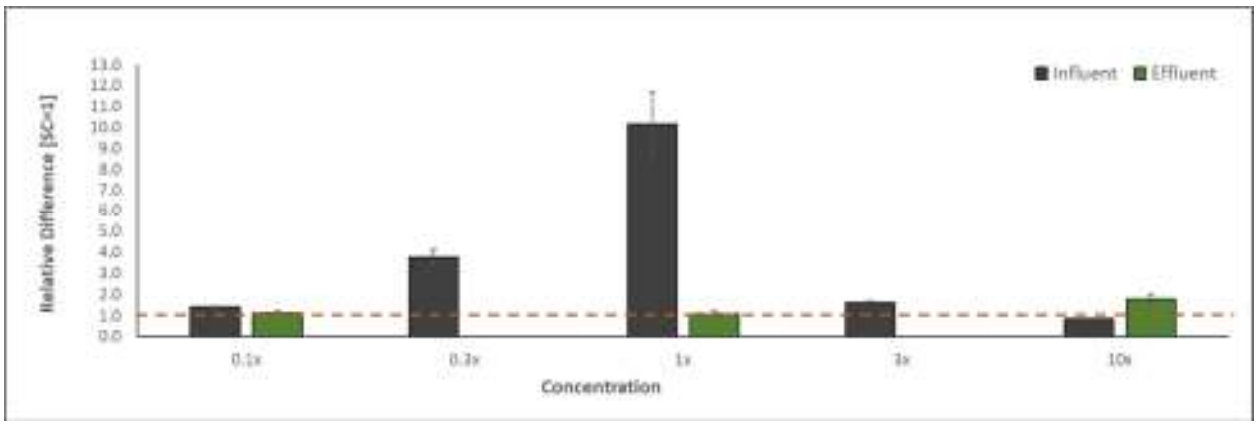

Figure A3.1 - Example of dose-response expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents at 0.1x, 0.3x, 1.0, 3.0x and 10x concentrations from Regina during spring determined by MDA-kb2 *in vitro* Assay. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

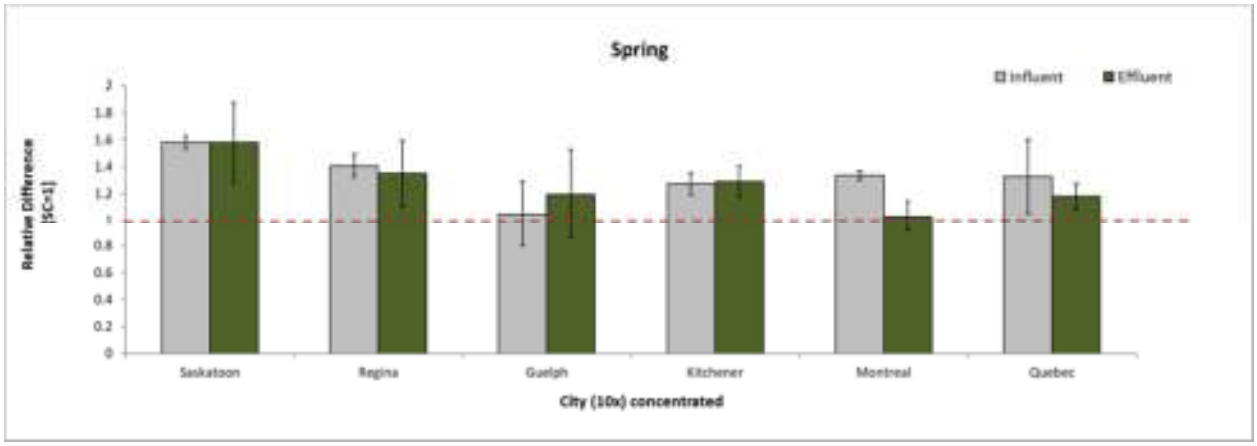

Figure A3.2 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MVLN *in vitro* Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

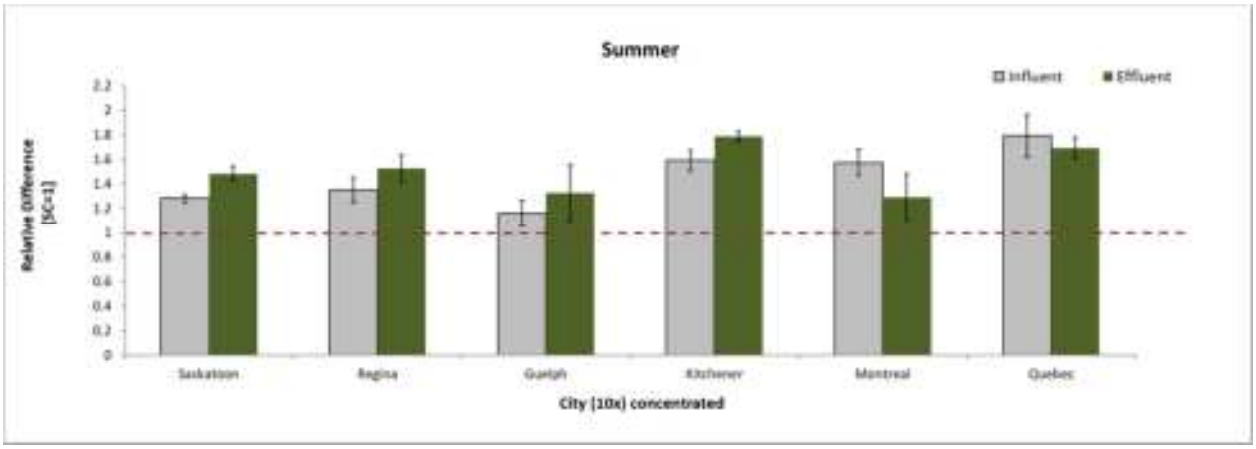

Figure A3.3 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MVLN *in vitro* Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

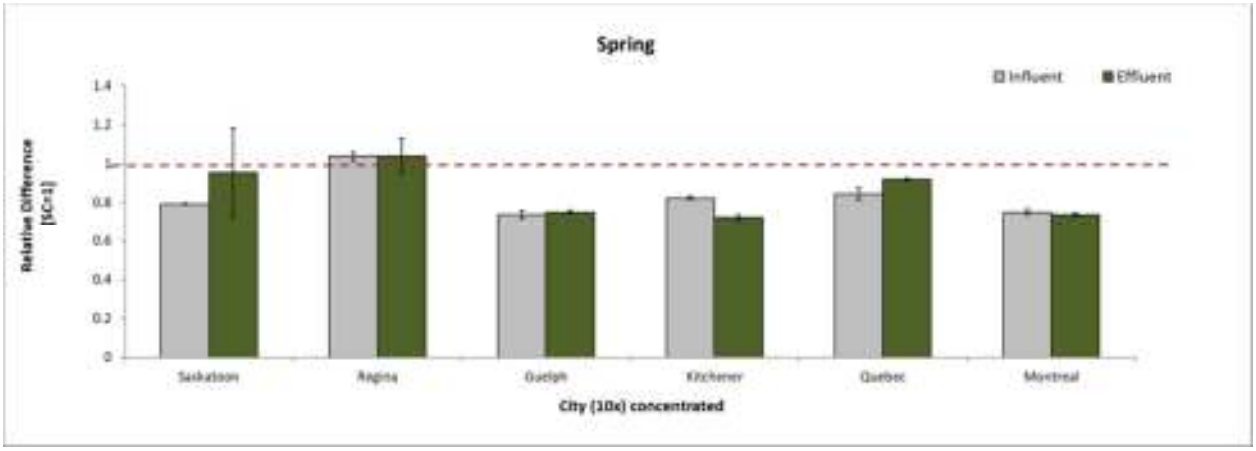

Figure A3.4 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MDA-kb2 *in vitro* Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

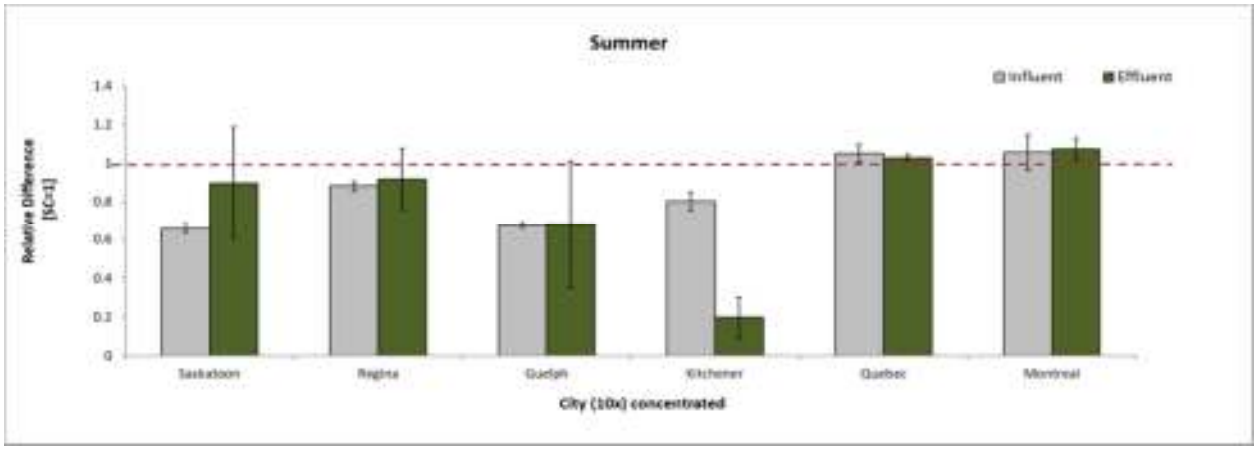

Figure A3.5 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by MDA-kb2 *in vitro* Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

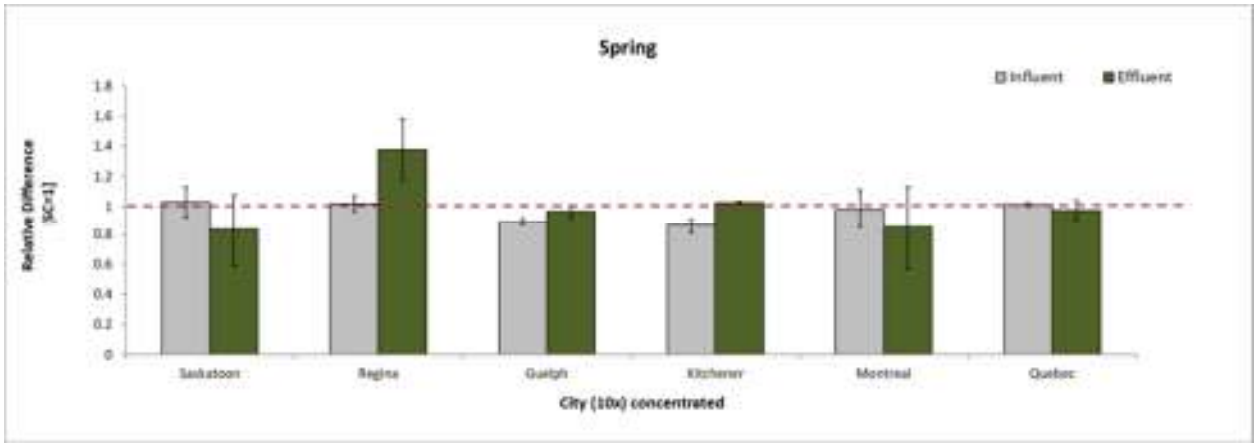

Figure A3.6 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by H295R *in vitro* Assay during spring of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

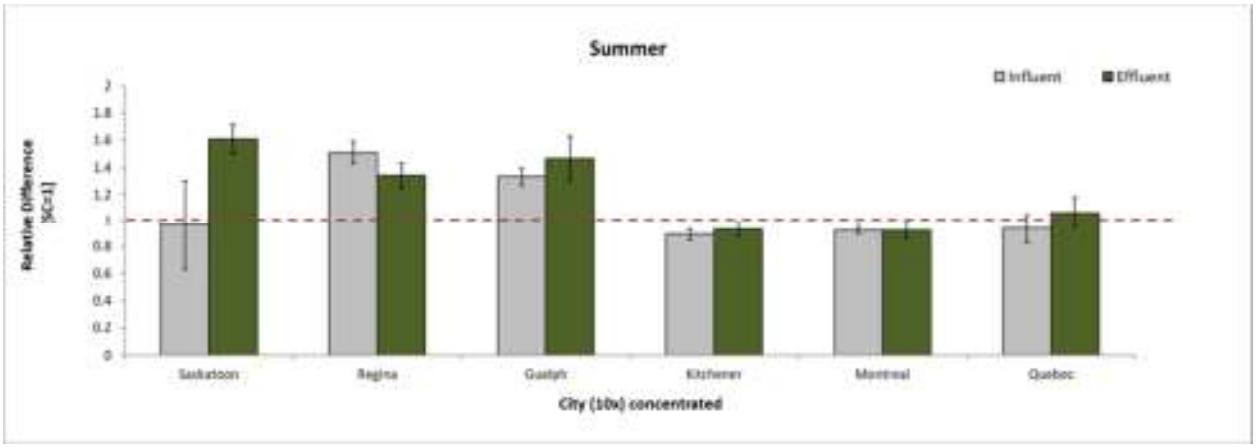

Figure A3.7 – Cytotoxicity assay expressed as relative changes compared to the solvent control (SC) of extracts of influents and effluents (10x concentrated) from six WWTPs determined by H295R *in vitro* Assay during summer of 2014. Results are represented as the mean ± SEM (n=4). Dashed line represents baseline (controls).

Table A2.1: Characteristics of Regina and Saskatoon WWTPs. Information obtained through a collaboration between WWTPs' personnel and the AIME project. Concentrations below than Method of detection limit (<MDL). (-) Data not available.

Endpoint	High concern	Low concern
Treatment plant	**Regina**	**Saskatoon**
Population Served	215,106	246,376
Treatment type	Primary – Lagoon system with phosphorous removal	Tertiary – with Biological nutrient removal (BNR), Conventional activated sludge
Primary treatment	Bar screen, grit removal and Primary clarifier	Bar screen, grit removal,and primary clarifier
Secondary treatment	Aeration, biological or bacterial removal of solids and other pollutants within lagoons	Biological nutrient removal (BNR), aeration and secondary clarifier.
Advanced treatment	Chemical phosphorus removal	Chemical phosphorus removal and nitrogen
Disinfection	Seasonal UV sterilization (April-November)	UV sterilization
Nitrification	No	Yes
Ammonia (mg/L)	1.2-41.2	4.5-42.6
Solids Retention Time	>30 d	<12 hours
Flow Capacity (m3/day) - Dry season	70,000	90,000
Flow Capacity (m3/day) - Rainfal/snow melt season	-	285,000
Flow (m3/day) - Maximum capacity	-	300,000
Geological Locations	Southern Saskatchewan	Central Saskatchewan
Upstream Watershed	Urban, Agricultural	Agricultural
Receiving Water	Wascana Creek	South Saskatchewan River
Dilution	Very low > 1%	High ~ 94 - 99%
Overflow	Bypasses Wascana Creek following special analysis	Treated
Caracteristics - Hospital Effluents?	Residential, commercial and hospitals	Yes - Residential, commercial and hospitals
Future upgrades	Build a new WWTP within 4 years to attend increased population and implemetation of nutrient removal process	None are planned

Table A2.2 – Summary of analytical results detected by Orbitrap Liquid Chromatography-Mass Spectrometry (LC-MS) expressed as ng/L in samples of influents and effluents from Regina and Saskatoon WWTPs during spring and summer of 2014 for 19 emergent contaminants.

Endpoint	Formula	Uses	Season	Regina		Saskatoon	
				Influent	Effluent	Influent	Effluent
Atrazine	C8H14ClN5	Herbicide	spring	<MDL	<MDL	0.1	0.1
			summer	1.1	0.1	0.5	0.2
Caffeine	C8H10N4O2	Stimulant	spring	135.9	9.7	62.6	<MDL
			summer	626.7	3.3	459.5	178.1
Carbamazepine	C15H12N2O	Anticonvulsant	spring	7.8	7.6	1.7	0.7
			summer	14.7	0.9	17.6	8.8
Clofibrate	C12H15ClO3	Antihyperlipidemic	spring	52.7	37.8	35.9	39.8
			summer	48.1	<MDL	43.5	30.9
DEET	C12H17NO	Insecticide	spring	671.1	395.5	152.1	10.3
			summer	7814.5	25.5	6702.1	3168.8
Diazepam	C16H13ClN2O	Benzodiazepine	spring	0.8	0.2	0.0	<MDL
			summer	<MDL	<MDL	0.1	0.1
EE2	C20H24O2	Ovulation inhibitor	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Estradiol	C18H24O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Estrone	C18H22O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Gemfibrozil	C15H22O3	Antihyperlipidemic	spring	<MDL	<MDL	<MDL	<MDL
			summer	268.2	<MDL	<MDL	<MDL
Ibuprofen	C13H18O2	Anti-inflammatory	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Naproxen	C14H14O3	Anti-inflammatory	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Pentachlorophenol	C6HCl5O	Pesticide	spring	<MDL	<MDL	<MDL	<MDL
			summer	1.1	<MDL	<MDL	<MDL
Progesterone	C21H30O2	Reproductive hormone	spring	2.3	<MDL	<MDL	<MDL
			summer	2.3	<MDL	1.8	<MDL
Testosterone	C19H28O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
Triclocarban	C13H9Cl3N2O	Antimicrobial disinfectant	spring	<MDL	<MDL	<MDL	<MDL
			summer	0.3	<MDL	<MDL	<MDL
Triclosan	C12H7Cl3O2	Antimicrobial disinfectant	spring	87.0	29.0	6.1	0.2
			summer	85.3	0.4	140.7	8.7
Trimethoprim	C14H18N4O3	Antibiotic	spring	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL
D3-Caffeine	-	Stimulant	spring	56.9	78.1	93.3	98.2
			summer	116.0	78.6	59.7	76.0

Table A2.3: Collection dates mean temperature (°C) of water and day.

Regina								
Day of Collection	Spring		Summer		Early Winter		Late Winter	
	°C Effluent	°C Day	°C Effluent	°C Day	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	2.0	-2.5	22.0	21.9	5.0	-8.2	5.0	-10.9
Day 2	4.0	-12.5	22.0	22.3	4.0	-9.3	2.0	-19.6
Day 3	2.0	-14.7	22.0	20.4	4.0	-11.2	2.0	-21.5
Day 4			22.0	21.2				
Mean	**2.7**	**-9.9**	**22.0**	**21.5**	**4.3**	**-9.6**	**3.0**	**-17.3**

Saskatoon								
Day of Collection	Spring		Summer		Early Winter		Late Winter	
	°C Effluent	°C Day	°C Effluent	°C Day	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	9.4	-13.9	17.50	21.7	14.30	-11.5	9.1	-8.0
Day 2	11.8	-11.0	17.60	20.0	13.00	-13.9	9.5	-16.0
Day 3					10.20	-22.2	9.4	-14.2
Mean	**10.6**	**-12.5**	**17.6**	**20.9**	**12.5**	**-15.9**	**9.3**	**-12.7**

Table A2.4: Occurrence of Carbamazepine, Clofibrate, DEET, and Triclosan in Influent and Effluents from WWTPs.

	Concentration ng/L				
Chemical	**Influent**	**Effluent**	**Location**	**Authors**	**Removal Efficiency**
Carbamazepine	325-1,850	940-1,510	Austria	Clara et al. (2005)	
		33–426	Canada	Metcalfe et al. (2003); Miao et al. (2003 and 2005); Gagne et al. (2006); Arlos et al. (2015)	
		60-108	China	Sui et at. (2010)	10-20% (Primary), Negative (Secondary Treatment)
		1075–6,300	Germany	Heberer (2002b); Hummel et al. (2006); Ternes et al. (2003)	8%
		291	Italy	Zuccato et al. (2004)	
	15-270	81–86	Japan	Okuda et al. (2008); Nakada et al. (2006, 2007 and 2008)	
	120-310		Mediterranean coast	Gómez et al. (2007)	
		73–729	South Korea	Kim et al. (2007); Choi et al. (2008); Behera et al. (2011)	
	<MDL-950		Spain	Gros et al. (2006)	
		290–960	Taiwan	Chen et al. (2008); Lin et al. (2005)	
		152–4,596	UK	Kasprzyk-Hordern et al. (2009); Zhang and Zhou (2007); Zhou et al. (2009)	
		33–270	USA	Guo and Krasner (2009); Spongberg and Witter (2008); Vanderford and Snyder (2006); Glassmeyer et al. (2005); Skadsen et al. (2004)	
Range	**<MDL-1,850**	**33-6,300**			**8-20%**

Chemical	Influent	Effluent	Location	Authors	Removal Efficiency
Clofibrate		37	Canada	Brun et al. (2006)	
			China	Sui et at. (2010)	<20% (Primary), <60% (Secondary Treatment)
			Germany	Ternes (1998); Heberer (2002b)	0-51%
	<MDL-110		Spain	Gros et al. (2006)	
	360	60-990	Switzerland	Tauxe-Wuersch et al. (2005); Tixier et al. (2003)	
Range	**<MDL-360**	**37-990**			**0-60%**
DEET	10-10,000	140	Australia	Costanzo et al. (2007); French et al. (2015)	
	600-1,200		China	Qian et al. (2010); Sui et at. (2010)	Negative (Primary), 65-85% (Secondary Treatment)
	3,000	1,500	Germany	Knepper et al. (2004)	
		88-2,238	Singapore	Tran et al. (2013)	20%-55%
		180-2,100	USA	Glassmeyer et al. (2005)	
Range	**10-10,000**	**140-2,238**			**20-85%**
Triclosan		23-434	Australia	Ying and Kookana (2007)	
	10-4,010	10-960	Canada	Lishman et al. (2006); Waiser et al. (2011); Arlos et al. (2015)	>95%
	1,200	10-600	Germany	Bester (2003 and 2005)	
	2,700-11,900	260-270	Japan	Nakada et al. (2008 and 2010)	
	380	160	Sweden	Bendz et al. (2005)	
		42-213	Switzerland	Singer et al. (2002)	
		340-3,100	UK	Kanda et al. (2003); Sabaliunas et al. (2003); Thompson et al. (2005)	>95%
	2,700-26,800	30-2,700	USA	McAvoy et al. (2002); Fair et al. (2009)	>95%
Range	**0-26,800**	**10-3,100**			**>95%**

Table A2.5: Extraction recovery (%) of spiked water samples from influent and effluent. Each sample was spiked in triplicate, average results are shown (n=3).

Chemicals	Influents (%)		Effluents (%)	
	Mean	SD	Mean	SD
Atrazine	0.0	0.0	0.0	0.0
Caffeine	44.3	10.5	9.8	17.0
Carbamazepine	270.8	116.7	285.7	68.0
DEET	369.4	110.0	0.0	0.0
EE2	0.0	0.0	18.6	5.5
Progesterone	17.5	16.3	70.0	5.7
Testosterone	74.5	8.4	34.4	4.7
Triclosan	48.6	14.8	70.2	3.3

Table A2.6 - Chemical standard used for Orbitrap analysis and sample spikes

Chemical Standard	Chemical Formula	Type	Supplier	CAS#
Ethinyl Estradiol	$C_{20}H_{24}O_2$	Hormone	Cambridge Isotope	57-63-6
Estradiol	$C_{18}H_{24}O_2$	Hormone	Cambridge Isotope	50-28-2
Estrone	$C_{18}H_{22}O_2$	Hormone	Cambridge Isotope	53-16-7
Progesterone	$C_{21}H_{30}O_2$	Hormone	Cambridge Isotope	57-83-0
Testosterone	$C_{19}H_{28}O_2$	Hormone	Cambridge Isotope	58-22-0
Atrazine	$C_8H_{14}ClN_5$	Pesticides	Sigma Aldrich	1912-24-9
Chlorpyrifos	$C_9H_{11}C_{l3}NO_3PS$	Pesticides	Sigma Aldrich	2921-88-2
Pentachlorophenol	$C_6HC_{l5}O$	Pesticides	Sigma Aldrich	87-86-5
Triclocarban	$C_{13}H_9C_{l3}N_2O$	Pesticides	Cambridge Isotope	101-20-2
Caffeine	$C_8H_{10}N_4O_2$	PPCP	Cambridge Isotope	58-08-2
Carbamazepine	$C_{15}H_{12}N_2O$	PPCP	Sigma Aldrich	298-46-4
Clofibrate	$C_{12}H_{15}ClO_3$	PPCP	Sigma Aldrich	637-07-0
DEET	$C_{12}H_{17}NO$	PPCP	Cambridge Isotope	132-62-3
Diazepam	$C_{16}H_{13}ClN_2O$	PPCP	Sigma Aldrich	439-14-5
Gemfibrozil	$C_{15}H_{18}O_2$	PPCP	Cambridge Isotope	25812-30-0
Ibuprofen	$C_{13}H_{18}O_2$	PPCP	Cambridge Isotope	15687-27-1
Miconazole nitrate	$C_{18}H_{14}Cl_4N_2O$	PPCP	Sigma Aldrich	22832-87-7
Naproxen	$C_{14}H_{14}O_3$	PPCP	Cambridge Isotope	22204-53-1
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	PPCP	Cambridge Isotope	723-46-6
Triclosan	$C_{12}H_7Cl_3O_2$	PPCP	Cambridge Isotope	3380-34-5
Trimethoprim	$C_{14}H_{18}N_4O_3$	PPCP	Sigma Aldrich	738-70-5

Table A3.1: Characteristics of all six WWTPs in Canada. Information obtained through a collaboration between WWTPs' personnel and the AIME project. Concentrations below than Method of detection limit (<MDL). Data not available (-).

Endpoint	Low concern			High concern		
Treatment plant	**Saskatoon**	**Guelph**	**Quebec City**	**Regina**	**Kitchener**	**Montreal**
Population Served	246,376	131,794	531,000	215,106	233,222	1,700,000
Treatment type	Tertiary – with Biological nutrient removal (BNR), Conventional activated sludge	Tertiary (Conventional and extended aeration activated sludge)	Secondary -Enhanced CPT, UV disinfection	Primary – Lagoon system with phosphorous removal	Secondary (Conventional activated sludge)	Primary - Lagoon with phosphorous removal
Primary treatment	Bar screen, grit removal,and primary clarifier	Bar screen, grit removal, primary clarifier, sedimentation	Bar screen, grit removal and primary clarifier	Bar screen, grit removal and Primary clarifier	Bar screen, grit removal, primary clarifier	Bar screen, grit removal, coagulant and polymer injection, sedimentation
Secondary treatment	Biological nutrient removal (BNR), aeration and secondary clarifier.	Aeration, secondary clarifier	Aeration, biofiltration and secondary clarifier	Aeration, biological or bacterial removal of solids and other pollutants within lagoons	Fine aeration bubbles and secondary clarifier	None
Advanced treatment	Chemical phosphorus removal and nitrogen	Rotating Bacterial Contactors, Sand filters	Enhanced Chemical phosphorus treatment	Chemical phosphorus removal	Chemical phosphorus removal	None
Disinfection	UV sterilization	Sodium hypochlorite (chlorination); Sodium Bisulphite (de-chlorination)	Seasonal UV sterilization (May-October)	Seasonal UV sterilization (April-November)	(Chlorination when UV system is not working properly)	None
Nitrification	Yes	Yes	No	No	Yes	No
Ammonia (mg/L)	4.5-42.6	0.1-1.5	5.0-25.0	1.2-41.2	13.4-29.1	Approx. 0.05 (non ionised)
Solids Retention Time	<12 hours	>10 d	-	>30 d	TP1 <2 - TP2 5.4 d	-
Flow Capacity (m3/day) - Dry season	90,000	45,942	388,000	70,000	65,858	2,500,000
Flow Capacity (m3/day) - Rainfal/snow melt season	285,000	-	-	-	-	7,600,000
Flow (m3/day) - Maximum capacity	300,000	-	1,200,000	-	-	-
Geological Locations	Central Saskatchewan	Southern Ontario	Appalachain province (type A8) Paleozoique (cambrian to inferior ordovician), Mudrock, conglomerate, sandstone & limestone	Southern Saskatchewan	Southern Ontario	St. Lawrence Platform, Paleozoic (Sup. ordovician - mid), Black river Trenton group, Limestone, shale, dolomite and sandstone.
Upstream Watershed	Agricultural	Urban	Suburban	Urban, Agricultural	Urban	Heavily Urbanized
Receiving Water	South Saskatchewan River	Speed River (a trib of the Grand River)	St. Lawrence River	Wascana Creek	Grand River	St. Lawrence River
Dilution	High ~ 94 - 99%	Lower <20%	High ~ 94 - 99%	Very low > 1%	Low 1-10%	-
Overflow	Treated	Treated	Retention basins with a total capacity of 142 000m3, overflow is mixed with rainwater and sent to the treatment plant. Excess is released, mixed, into local waters	Bypasses Wascana Creek following special analysis	Treated	Not treated discharged into the St. Lawrence River
Caracteristics - Hospital Effluents?	Yes - Residential, commercial and hospitals	-	Yes - 4 mean of 2,141 m3/day	Residential, commercial and hospitals	-	Yes - 25 hospitals
Future upgrades	None are planned	Membrane Bioreactors, TP removal	None are planned	Build a new WWTP within 4 years to attend increased population and implemetation of nutrient removal process	Nitrification, filtration	Add a terminal disinfection process using ozone (capacity of 40 m3/seconds)

Table A3.2 – Summary of analytical results detected by Orbitrap LC-MS expressed as ng/L in samples of influents and effluents from six WWTPs across Canada during spring and summer of 2014 for 19 emergent contaminants. Concentrations below the method detection limit (<MDL). Samples not available for chemicals analysis (N/A).

Endpoint	Formula	Uses	Season	Saskatoon		Regina		Guelph		Kitchener		Quebec		Montreal	
				Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Atrazine	C8H14ClN5	Herbicide	spring	0.09	0.06	<MDL	<MDL	6.06	0.33	1.93	0.61	<MDL	0.23	0.73	10.11
			summer	0.52	0.16	1.06	0.08	0.08	0.21	22.09	N/A	<MDL	<MDL	<MDL	1.05
Caffeine	C8H10N4O2	Stimulant	spring	62.56	<MDL	135.85	9.74	215.38	6.29	751.39	<MDL	3.57	288.33	20.93	660.90
			summer	459.50	178.06	626.70	3.27	252.41	2.49	1,033.42	N/A	354.19	660.70	<MDL	368.64
Carbamazepine	C15H12N2O	Anticonvulsant	spring	1.66	0.65	7.84	7.62	5.07	18.90	2.25	1.24	0.05	15.86	1.22	5.33
			summer	17.61	8.79	14.70	0.91	5.82	16.55	29.90	N/A	0.47	2.93	<MDL	0.54
Clofibrate	C12H15ClO3	Antihyperlipidemic	spring	35.90	39.82	52.73	37.80	<MDL	109.35	63.68	<MDL	<MDL	90.77	27.89	<MDL
			summer	43.49	30.93	48.14	<MDL	31.20	78.96	<MDL	N/A	<MDL	<MDL	<MDL	<MDL
DEET	C12H17NO	Insecticide	spring	152.12	10.29	671.07	395.45	179.27	74.38	70.28	7.68	3.15	168.15	56.21	6.98
			summer	6,702.12	3,168.79	7,814.49	25.54	1,603.63	61.14	3,161.11	N/A	28.25	113.70	<MDL	34.41
Diazepam	C16H13ClN2O	Benzodiazepine	spring	0.03	<MDL	0.82	0.22	<MDL	<MDL	<MDL	<MDL	<MDL	0.48	<MDL	0.08
			summer	0.06	0.11	<MDL	<MDL	0.04	<MDL	<MDL	N/A	<MDL	<MDL	<MDL	<MDL
EE2	C20H24O2	Ovulation inhibitor	spring	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	N/A	<MDL	<MDL	<MDL	<MDL
Estradiol	C18H24O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	N/A	<MDL	<MDL	<MDL	<MDL
Estrone	C18H22O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	N/A	<MDL	<MDL	<MDL	<MDL
Gemfibrozil	C15H22O3	Antihyperlipidemic	spring	<MDL	<MDL	<MDL	<MDL	23.13	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	16.40
			summer	<MDL	<MDL	268.22	<MDL	<MDL	<MDL	<MDL	N/A	<MDL	17.31	<MDL	<MDL
Ibuprofen	C13H18O2	Anti-inflammatory	spring	<MDL	<MDL	<MDL	<MDL	8,813.05	<MDL	<MDL	<MDL	202.72	<MDL	<MDL	687.90
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	41,618.64	N/A	543.31	1,637.31	374.47	194.13
Naproxen	C14H14O3	Anti-inflammatory	spring	<MDL	<MDL	<MDL	<MDL	895.16	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	18.73
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	11,446.64	N/A	72.20	320.99	17.32	<MDL
Pentachlorophenol	C6HCl5O	Pesticide	spring	<MDL	<MDL	<MDL	<MDL	4.59	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.36
			summer	<MDL	<MDL	1.08	<MDL	<MDL	<MDL	1.45	N/A	0.08	1.12	<MDL	1.26
Progesterone	C21H30O2	Reproductive hormone	spring	<MDL	<MDL	2.31	<MDL	13.05	<MDL	1.58	<MDL	<MDL	<MDL	0.87	7.47
			summer	1.78	<MDL	2.31	<MDL	0.62	<MDL	9.32	N/A	<MDL	<MDL	<MDL	<MDL
Testosterone	C19H28O2	Reproductive hormone	spring	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.58
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	93.44	N/A	<MDL	<MDL	<MDL	<MDL
Triclocarban	C13H9Cl3N2O	Antimicrobial disinfectant	spring	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.08
			summer	<MDL	<MDL	0.26	<MDL	<MDL	<MDL	<MDL	N/A	<MDL	<MDL	0.91	<MDL
Triclosan	C12H7Cl3O2	Antimicrobial disinfectant	spring	6.06	0.23	87.03	28.95	60.51	3.79	24.11	1.76	7.13	53.36	7.24	62.63
			summer	140.74	8.74	85.33	0.37	34.07	2.27	1,427.69	N/A	15.68	106.08	1.52	13.93
Trimethoprim	C14H18N4O3	Antibiotic	spring	<MDL	<MDL	<MDL	<MDL	2.26	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.75
			summer	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.31	N/A	0.39	0.71	<MDL	0.90
D3-Caffeine	-	Stimulant	spring	93.30	98.20	56.90	78.10	79.60	94.50	65.10	20.50	42.60	38.30	59.70	82.70
			summer	59.70	76.00	116.00	78.60	66.10	89.30	92.80	N/A	74.00	38.30	86.10	59.20

Table A3.3: Extraction recovery (%) of spiked water samples from influent and effluent. Each sample was spiked in triplicate, average results are shown (n=3).

Chemicals	Influents (%)		Effluents (%)	
	Mean	SD	Mean	SD
Atrazine	0.0	0.0	0.0	0.0
Caffeine	44.3	10.5	9.8	17.0
Carbamazepine	270.8	116.7	285.7	68.0
DEET	369.4	110.0	0.0	0.0
EE2	0.0	0.0	18.6	5.5
Progesterone	17.5	16.3	70.0	5.7
Testosterone	74.5	8.4	34.4	4.7
Triclosan	48.6	14.8	70.2	3.3

Table A3.4 - Chemical standard used for Orbitrap analysis and sample spikes

Chemical Standard	Chemical Formula	Type	Supplier	CAS#
Ethinyl Estradiol	$C_{20}H_{24}O_2$	Hormone	Cambridge Isotope	57-63-6
Estradiol	$C_{18}H_{24}O_2$	Hormone	Cambridge Isotope	50-28-2
Estrone	$C_{18}H_{22}O_2$	Hormone	Cambridge Isotope	53-16-7
Progesterone	$C_{21}H_{30}O_2$	Hormone	Cambridge Isotope	57-83-0
Testosterone	$C_{19}H_{28}O_2$	Hormone	Cambridge Isotope	58-22-0
Atrazine	$C_8H_{14}ClN_5$	Pesticides	Sigma Aldrich	1912-24-9
Chlorpyrifos	$C_9H_{11}C_{l3}NO_3PS$	Pesticides	Sigma Aldrich	2921-88-2
Pentachlorophenol	$C_6HC_{l5}O$	Pesticides	Sigma Aldrich	87-86-5
Triclocarban	$C_{13}H_9C_{l3}N_2O$	Pesticides	Cambridge Isotope	101-20-2
Caffeine	$C_8H_{10}N_4O_2$	PPCP	Cambridge Isotope	58-08-2
Carbamazepine	$C_{15}H_{12}N_2O$	PPCP	Sigma Aldrich	298-46-4
Clofibrate	$C_{12}H_{15}ClO_3$	PPCP	Sigma Aldrich	637-07-0
DEET	$C_{12}H_{17}NO$	PPCP	Cambridge Isotope	132-62-3
Diazepam	$C_{16}H_{13}ClN_2O$	PPCP	Sigma Aldrich	439-14-5
Gemfibrozil	$C_{15}H_{18}O_2$	PPCP	Cambridge Isotope	25812-30-0
Ibuprofen	$C_{13}H_{18}O_2$	PPCP	Cambridge Isotope	15687-27-1
Miconazole nitrate	$C_{18}H_{14}Cl_4N_2O$	PPCP	Sigma Aldrich	22832-87-7
Naproxen	$C_{14}H_{14}O_3$	PPCP	Cambridge Isotope	22204-53-1
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	PPCP	Cambridge Isotope	723-46-6
Triclosan	$C_{12}H_7Cl_3O_2$	PPCP	Cambridge Isotope	3380-34-5
Trimethoprim	$C_{14}H_{18}N_4O_3$	PPCP	Sigma Aldrich	738-70-5

Table A3.5: Occurrence of Carbamazepine, Clofibrate, DEET, Gemfibrozil, Ibuprofen, Naproxen, Progesterone, Testosterone, and Triclosan in Influent and Effluents from WWTPs.

	Concentration ng/L			
Chemical	**Influent**	**Effluent**	**Location**	**Authors**
Carbamazepine	325-1,850	940-1,510	Austria	Clara et al. (2005)
		33–426	Canada	Metcalfe et al. (2003); Miao et al. (2003 and 2005); Gagne et al. (2006); Arlos et al. (2015)
		60-108	China	Sui et at. (2010)
		1075–6,300	Germany	Heberer (2002b); Hummel et al. (2006); Ternes et al. (2003)
		291	Italy	Zuccato et al. (2004)
	15-270	81–86	Japan	Okuda et al. (2008); Nakada et al. (2006, 2007 and 2008)
	120-310		Mediterranean coast	Gómez et al. (2007)
		73–729	South Korea	Kim et al. (2007); Choi et al. (2008); Behera et al. (2011)
	<MDL-950		Spain	Gros et al. (2006)
		290–960	Taiwan	Chen et al. (2008); Lin et al. (2005)
		152–4,596	UK	Kasprzyk-Hordern et al. (2009); Zhang and Zhou (2007); Zhou et al. (2009)
		33–270	USA	Guo and Krasner (2009); Spongberg and Witter (2008); Vanderford and Snyder (2006); Glassmeyer et al. (2005); Skadsen et al. (2004)
Range	**<MDL-1,850**	**33-6,300**		
Range (Current study)	**0.05-29.90**	**0.54-18.90**		

Chemical	Influent	Effluent	Location	Authors
Clofibrate		37	Canada	Brun et al. (2006)
			China	Sui et at. (2010)
			Germany	Ternes (1998); Heberer (2002b)
	<MDL-110		Spain	Gros et al. (2006)
	360	60-990	Switzerland	Tauxe-Wuersch et al. (2005); Tixier et al. (2003)
Range	**<MDL-360**	**37-990**		
Range (Current study)	**27.89-63.38**	**30.93-109.35**		

Chemical	Influent	Effluent	Location	Authors
DEET	10-10,000	140	Australia	Costanzo et al. (2007); French et al. (2015)
	600-1,200		China	Qian et al. (2010); Sui et at. (2010)
	3,000	1,500	Germany	Knepper et al. (2004)
		88-2,238	Singapore	Tran et al. (2013)
		180-2,100	USA	Glassmeyer et al. (2005)
Range	**10-10,000**	**140-2,238**		
Range (Current study)	**3.15-7,814**	**6.98-3,168**		

Chemical	Influent	Effluent	Location	Authors
Gemfibrozil	2,000-3,800	>MDL-2,800	Australia	Ying et al. (2009)
		59-3,380	Canada	Metcalfe et al. (2003b); Gagne et al. (2006); Waiser et al. (2011b)
		4,760	Europe	Andreozzi et al., 2003
		60-1,350	France	Quinn et al. (2008)
		0.71	Greece	Quinn et al. (2008)
		810-4,760	Italy	Quinn et al. (2008)
		1,000-6,800	Spain	Bueno et al. (2012)
Range	**2,000-3,800**	**0.71-6,800**		
Range (Current study)	**23.13 and 268.22**	**16.40 and 17.31**		

Chemical	Influent	Effluent	Location	Authors
Ibuprofen		384-4,000	Canada	Metcalfe et al. (2003); Lishman et al. (2006); Zorita et al. (2009)
	3,350		Germany	Ternes et al. (1998); Stumpf et al. (1996)
			Japan	Nakada et al. (2006)
	14,000	5,000	Spain	Bueno et al. (2012)
		1,300	Switzerland	Tixier et al. (2003)
Range	**3,350-14,000**	**384-5,000**		
Range (Current study)	**202.72-41,618**	**194.13-1,637**		

Chemical	Influent	Effluent	Location	Authors
Naproxen	600		Brazil	Daughton and Ternes (1999)
		7,600	Canada	Waiser et al. (2011b)
		520	Germany	Daughton and Ternes (1999)
	11,000		Italy	Verlicchi et al. (2012)
		1,500	Spain	Bueno et al. (2012)
	3,700	2,500	Sweden	Bendz et al. (2005)
		2,600	Switzerland	Tixier et al. (2003)
Range	**600-11,000**	**520-7,600**		
Range (Current study)	**17.32-11,446**	**18-73 and 320.99**		

Chemical	Influent	Effluent	Location	Authors
Progesterone		1.5-16.9	Europe	Vulliet et al. (2007)
		0.2-100	Belgium	Pauwels et al. (2008)
		408	South Africa	Manickum and John (2013)
	72.9-74.2	<MDL	USA	Esperanza et al., (2007)
Range	**72.9-74.2**	**<MDL-408**		
Range (Current study)	**0.62-13.05**	**7.47**	(I) Guelph influent samples from summer and spring, (E) only in Montreal during spring	

Chemical	Influent	Effluent	Location	Authors
Testosterone	24-180	<1	Canada	Lee et al. (2004)
	343	11	South Africa	Manickum and John (2013)
	89.5	<MDL	USA	Esperanza et al., (2007)
Range	**24-343**	**<MDL-11**		
Range (Current study)	**93.44**	**1.58**		

Chemical	Influent	Effluent	Location	Authors
Triclosan		23-434	Australia	Ying and Kookana (2007)
	10-4,010	10-960	Canada	Lishman et al. (2006); Waiser et al. (2011); Arlos et al. (2015)
	1,200	10-600	Germany	Bester (2003 and 2005)
	2,700-11,900	260-270	Japan	Nakada et al. (2008 and 2010)
	380	160	Sweden	Bendz et al. (2005)
		42-213	Switzerland	Singer et al. (2002)
		340-3,100	UK	Kanda et al. (2003); Sabaliunas et al. (2003); Thompson et al. (2005)
	2,700-26,800	30-2,700	USA	McAvoy et al. (2002); Fair et al. (2009)
Range	**0-26,800**	**10-3,100**		
Range (Current study)	**1.52-1,427**	**0.37-106.08**		

Table A3.6: Collection dates mean temperature (°C) of water and day from six WWTPs.

Saskatoon				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	9.4	-13.9	17.50	21.7
Day 2	11.8	-11.0	17.60	20.0
Mean	**10.6**	**-12.5**	**17.6**	**20.9**

Regina				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	2.0	-2.5	22.0	21.9
Day 2	4.0	-12.5	22.0	22.3
Day 3	2.0	-14.7	22.0	20.4
Day 4			22.0	21.2
Mean	**2.7**	**-9.9**	**22.0**	**21.5**

Gueph				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	Not available	9.7	Not available	19.5
Day 2			Not available	19.4
Day 3				
Day 4				
Mean		**9.7**		**19.5**

Kitchener				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	Not available	4.7	Not available	15.0
Day 2			Not available	13.4
Day 3				
Day 4				
Mean		**4.7**		**14.2**

Quebec				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	9.4	-10.8	19.2	12.9
Day 2			19.7	16.7
Day 3			18.2	15.7
Mean	**9.4**	**-10.8**	**19.0**	**15.1**

Montreal				
Day of Collection	Spring		Summer	
	°C Effluent	°C Day	°C Effluent	°C Day
Day 1	7.3	-6.3	20.6	7.8
Day 2			20.6	12.6
Mean	**7.3**	**-6.3**	**20.6**	**10.2**